



universität  
wien

# DIPLOMARBEIT

Titel der Diplomarbeit

„Characterization of MTP during chicken embryonic development“

Verfasserin

Christine Eresheim

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Wien, 2009

Studienkennzahl lt. Studienblatt:

A 490

Studienrichtung lt. Studienblatt:

Molekulare Biologie

Betreuerin:

Ao. Prof. DI Dr. Marcela Hermann



## Danke!

Nun ist es endlich soweit! Ich bin beim Dankeschreiben meiner Diplomarbeit angelangt. Der Weg hierher war ein langer – zwischendurch oft mühsam, aber auch sehr bereichernd. Meine Erinnerungen an die anfänglichen (Über-)Forderungen bringen mich heute zum Schmunzeln und umso glücklicher macht es mich, dass sich ein weiterer Abschnitt meiner Ausbildung bzw. meines Lebens dem Ende zuneigt. Danke an viele liebe Menschen, die mich in dieser Zeit begleitet, unterstützt und gefördert haben!

Besonders danken möchte ich Marcela, die mich in ihre Gruppe aufgenommen hat und mir somit ermöglichte, meine Diplomarbeit bei ihr zu schreiben und die mir währenddessen mit Rat und Tat zur Seite stand. Danke auch an Erwin, der mir bei so manchen fachlichen Fragen weiterhalf! Desiree und Julia, danke für eure Hilfe jeglicher Art und für die vielen lustigen Stunden, die ich mit euch und den Schneiders verbrachte!

Dankbarkeit anderer Art gebührt meinen Eltern! Sie beide haben mich zu jeder Zeit in meinen Entscheidungen unterstützt und mich wieder aufgebaut, wenn's mal nicht so gut lief. Aber auch ohne ihre finanzielle Unterstützung wären meine letzten Jahre nicht so verlaufen, wie sie waren. Danke!

Kathi, auch dir danke, dafür, dass du immer für mich da bist und mich in meiner Ansicht, dass du die beste Schwester der Welt bist, stärkst!

Basti, dir bin ich besonders dankbar! Ohne dich hätte ich es wohl nicht durchgehalten bis hierher. Du warst und bist immer für mich da und somit eine wesentliche Stütze in meinem Leben, egal wie viele Kilometer zwischen uns liegen. Danke!

---

## Contents

Abstract.....	5
Zusammenfassung.....	7
1. Introduction.....	9
1.1. The large lipid transfer protein superfamily .....	9
1.2. Structure, localization, functions and regulation of MTP .....	11
1.2.1. Structure and localization of MTP .....	11
1.2.3. Functions of MTP .....	14
1.2.4. Regulation of MTP .....	14
1.3. MTP in humans.....	15
1.3.1. Abetalipoproteinemia.....	15
1.3.2. MTP as a target in hyperlipidemias .....	16
1.3.3. MTP during human embryonic development .....	17
1.4. MTP during mouse embryonic development.....	18
1.5. Chicken MTP .....	19
1.6. Embryonic development of the chicken .....	20
1.6.1. Oocyte formation and fertilization.....	20
1.6.2. Early embryonic development .....	21

---

1.6.4. The yolk sac .....	22
1.6.5. The liver .....	23
1.6.6. The gastrointestinal system.....	24
1.6.7. The kidney .....	24
1.6.8. Late embryonic development and hatching .....	24
1.7. The role of EECs.....	25
1.8. Aim of the thesis .....	26
2. Materials and Methods.....	27
2.1. Chemicals and enzymes.....	27
2.2. Bacterial strains and vector systems .....	27
2.3. Animals .....	29
2.4. Oligonucleotide primers.....	29
2.5. Antibodies .....	30
2.6. Molecular biological methods: DNA.....	30
2.6.1. cDNA synthesis .....	30
2.6.2. Polymerase chain reaction (PCR) .....	31
2.6.3. DNA gel electrophoresis.....	32
2.6.4. DNA gel extraction with Xact DNA gel extraction kit from GenXpress .....	32
2.6.5. T/A-cloning.....	33
2.6.6. Transformation of competent <i>Escherichia coli</i> ( <i>E.coli</i> ) cells .....	33
2.6.7. Mini preparation of plasmid DNA .....	34
2.6.8. DNA sequencing.....	35
2.6.9. Quantitative real-time PCR.....	35
2.7. Molecular biological methods: RNA.....	36
2.7.1. Isolation of total RNA.....	36
2.7.2. Determination of RNA concentration.....	37
2.8. Molecular biological methods: protein .....	37
2.8.1. Preparation of membrane protein extracts .....	37
2.8.2. Determination of protein concentration .....	38
2.8.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) .....	39
2.8.4. Coomassie stain .....	41

---

---

2.8.5. Western blot analysis .....	41
2.8.6. Pulse chase .....	43
2.8.7. Immunoprecipitation .....	45
2.8.8. SDS-PAGE and detection .....	45
2.9. Histological methods .....	46
2.9.1. Paraffinsections .....	46
2.9.2. Cryosections .....	47
2.10. Cell and Tissue Culture .....	49
2.10.1 Isolation of endodermal epithelial cells (EECs) .....	49
2.10.2. Cysteine lyase activity-assay .....	50
2.10.3. Tissue culture .....	51
3. Results .....	52
3.1. Expression of MTP in chicken extraembryonic tissue .....	52
3.1.1. Western blot analysis .....	52
3.1.2. Quantitative PCR .....	54
3.1.3. Immunohistochemistry .....	55
3.1.4. Cell culture of and MTP expression in EECs .....	57
3.1.5. Organ culture and pulse chase analysis .....	57
3.1.6. EEC cell lysate .....	58
3.2. Expression of MTP in chicken embryonic tissues .....	60
3.2.1. Liver .....	60
3.2.2. Small intestine .....	63
3.2.3. Kidney .....	65
3.3. MTP mRNA and protein levels in various tissues at different developmental stages of the chicken .....	69
3.3.1. Transcript levels .....	69
3.3.2. Protein levels .....	71
4. Discussion .....	72
Bibliography .....	79
List of figures .....	83

---

---

List of tables.....	85
Abbreviations .....	86
Curriculum vitae .....	90

---

## Abstract

The microsomal triglyceride transfer protein (MTP) is a protein that plays a major role in the synthesis of apolipoprotein B-containing lipoproteins. It is located in the lumen of the endoplasmic reticulum (ER) and is involved in the loading of nascent lipoproteins with lipids. In humans, MTP has been shown to be involved in the biogenesis of apolipoprotein B100 in the liver and apolipoprotein B48 in the small intestine. MTP and apoB are also expressed in the human placenta, suggesting that this organ is also able to synthesize and secrete apoB-containing lipoproteins. During mouse embryonic development, MTP is expressed in the yolk sac, as well as in liver and small intestine. MTP was not detected in cardiac or renal tissue of mouse embryos. In the laying hen, it has been shown that MTP is expressed in liver, small intestine, and kidney. Chicken liver synthesizes and secretes also apoB100-containing lipoproteins, but the small intestine lacks apoB48. Instead of apoB48, chicken small intestine secretes portomicrons, which are apoB100-containing lipoproteins.

As MTP is expressed in the yolk sac of mouse, in the human placenta, and in various tissues involved in lipid metabolism in the laying hen, it could be assumed that MTP is also present in the yolk sac of the developing chicken embryo. The chicken yolk sac is responsible for the supply of the growing embryo with nutrients contained in the yolk. Using quantitative PCR and western blot analysis, it could be shown here that MTP is expressed in the yolk sac, its expression starting at the end of the first trimester of

---

embryonic development, and it is still present in the yolk sac in the early post-natal period. Immunohistochemistry and western blot analysis indicated that MTP is expressed in the inner layer of the yolk sac, the endodermal epithelial cells (EECs). Further, pulse chase analysis showed that apoB100 is synthesized and secreted by cultured yolk sac. It is therefore likely that MTP plays an important role in de novo synthesis of lipoproteins in this organ. In addition to the yolk sac, liver, small intestine, and kidney of chicken embryos at different developmental stages were included into the analysis on MTP expression. In liver and kidney of chicken embryos, MTP mRNA and protein could be detected from the middle of the second trimester of embryonic development on. MTP expression in the chicken small intestine starts in the third trimester of embryonic development. Immunohistochemistry of chicken embryonic liver, small intestine, and kidney confirmed the presence of MTP in the respective tissues and also gave insights into the localization of MTP in these tissues.

---

## Zusammenfassung

Das mikrosomale Triglycerid Transfer-Protein (MTP) ist ein Proteinkomplex, der bei der Synthese von ApolipoproteinB-enthaltenden Lipoproteinen eine wichtige Rolle spielt. MTP befindet sich im Lumen des Endoplasmatischen Reticulums (ER) und ist in die Beladung von entstehenden Lipoproteinen mit Lipiden involviert. Es wurde gezeigt, dass MTP an der Biogenese von ApolipoproteinB100 in der Leber und von ApolipoproteinB48 im Dünndarm des Menschen beteiligt ist. MTP und apoB werden auch in der humanen Plazenta exprimiert, was ein Hinweis darauf ist, dass dieses Organ auch fähig ist, apoB-enthaltende Lipoproteine zu synthetisieren und zu sekretieren. Während der Embryonalentwicklung von Mäusen wird MTP im Dottersack exprimiert. Weiters ist es auch in der Leber und im Dünndarm von Mausembryonen vorhanden, konnte allerdings weder in deren Herz- noch Nierengewebe nachgewiesen werden. In der Legehenne wurde gezeigt, dass MTP in der Leber, im Dünndarm und in der Niere exprimiert wird. Die Leber von Hühnern synthetisiert und sekretiert apoB100-enthaltende Lipoproteine, aber dem Dünndarm fehlt apoB48. Statt apoB48 sekretiert der Dünndarm von Hühnern Portomicronen, die auch zu den apoB100-enthaltenden Lipoproteinen gehören.

Da MTP im Dottersack von Mäusen, in der menschlichen Plazenta und in verschiedenen, in den Lipidmetabolismus involvierten Geweben der Legehenne exprimiert ist, konnte



---

vermutet werden, dass MTP auch im Dottersack des sich entwickelnden Hühnerembryos vorhanden ist. Der Dottersack von Hühnern ist verantwortlich für die Versorgung des wachsenden Embryos mit Nährstoffen, die der Dotter enthält. Mittels quantitativer PCR und Western Blot Analyse konnte hier gezeigt werden, dass MTP vom Ende des ersten Trimesters der Embryonalentwicklung an im Dottersack exprimiert ist. Aber auch in den ersten Tagen nach dem Schlüpfen ist MTP im Dottersack noch vorhanden. Immunhistochemie und Western Blot Analyse ergaben Hinweise darauf, dass MTP in der inneren Schicht des Dottersacks, den endodermalen epithelialen Zellen (EECs) exprimiert wird. Weiters konnte mit Pulse Chase Experimenten gezeigt werden, dass kultivierte Dottersack-Stücke apoB synthetisieren und sekretieren. Dieser Befund legt nahe, dass MTP eine wichtige Rolle in der de novo Synthese von Lipoproteinen im Dottersack spielt. Neben dem Dottersack wurden auch Leber, Dünndarm und Niere von Hühnerembryonen verschiedener Entwicklungsstadien in die Untersuchungen der MTP-Expression miteinbezogen. In der Leber und der Niere von Hühnerembryonen konnten MTP mRNA und Protein von der Mitte des zweiten Trimesters der Embryonalentwicklung an detektiert werden. Im Dünndarm beginnt die Expression von MTP im dritten Trimester der Embryonalentwicklung. Immunhistochemische Analysen von Leber, Dünndarm und Niere von Hühnerembryonen bestätigten die Expression von MTP im jeweiligen Gewebe und gaben teilweise auch Aufschlüsse über die Lokalisation von MTP in diesen Geweben.

---

## 1. Introduction

### 1.1. The large lipid transfer protein superfamily

The microsomal triglyceride transfer protein (MTP) is a member of the large lipid transfer (LLT) protein (LLTP) superfamily. Proteins belonging to the LLTP superfamily are involved in mediating the circulatory lipid transport in animals. In addition to MTP (or MTP-like LLTPs), there are two more major families of LLTPs, namely apolipoproteinB (apoB)-like LLTPs and vitellogenin (Vtg)-like LLTPs. These proteins vary in their major lipid binding sites (LLT module and amphipatic secondary structures) and are therefore able to bind hundreds of different lipid molecules.

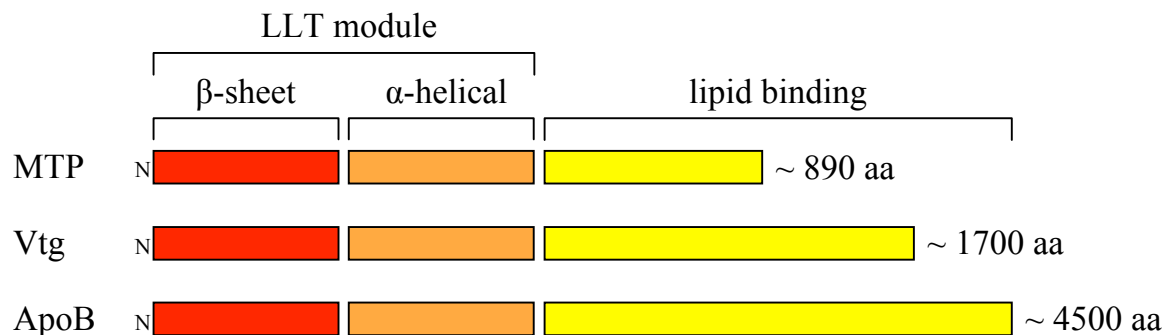
Beside common characteristics, such as lipid transport, each family has different functions:

- ApoB is present in vertebrates and it is responsible for postprandial lipid uptake and lipid redistribution in the body.
- Vtg is the major yolk protein found in females of most egg-laying animals, non-mammalian vertebrates and invertebrates and supplies the developing oocyte with nutrients, including lipids.

- 
- MTP is present in vertebrates and invertebrates, and has been shown to enhance the biosynthesis of other LLTPs, as demonstrated for apolipoproteins and Vtg. (Smolenaars et al. 2007)

LLTPs seem to be limited to animals, leading to the suggestion that this superfamily evolved at the beginning of animal evolution. Vertebrates encode MTP, apoB, and, except in mammals, one or more Vtgs, thus humans have only apoB and MTP. (Smolenaars et al. 2007)

All members of the LLTP superfamily share the LLT module, which is located at the N terminus of almost every LLTP. Apart from this LLT module, LLTPs vary to a great extent in size and organization. The smallest of the LLTPs is MTP with around 890 amino acids (aa), whereas other LLTPs are made up of up to 4.500 aa (apoB). (Figure 1.1)



**Figure 1.1. Members of the LLTP superfamily.**

This figure illustrates the structural organization of LLTPs, showing that the LLT module is located at the N terminus of the proteins and that the lipid binding domain vary to a great extent in size. (adapted from Shelness and Ledford. 2005)

The LLT module is made up of four major structural elements in all LLTPs (Figure 1.2.A) These four elements consist of one  $\alpha$ -helical bundle and three  $\beta$ -sheets, which are arranged in a way to form binding sites for homodimerization (Vtg) and heterodimerization (MTP with protein disulfide isomerase (PDI) and apoB) as well as lipid binding and lipid transfer. (Smolenaars et al. 2007)

---

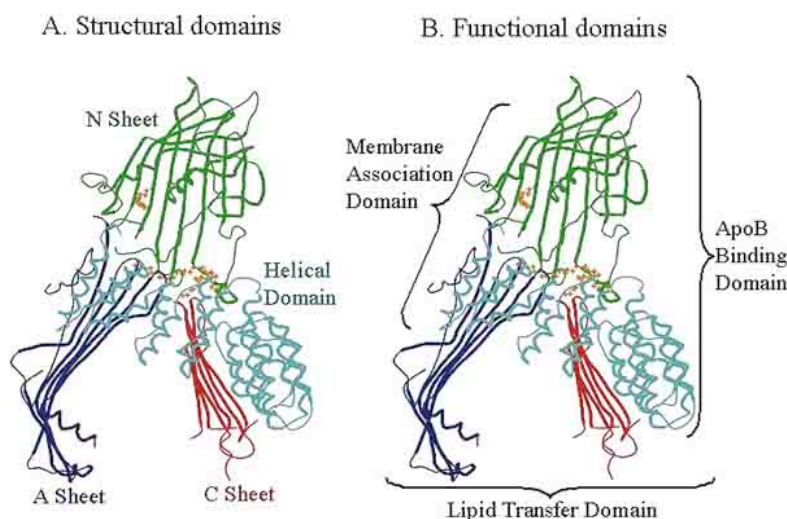
## 1.2. Structure, localization, functions and regulation of MTP

### 1.2.1. Structure and localization of MTP

It was shown that active MTP consists of two non-covalently bound polypeptides, which are the 97 kDa M-subunit and the 55 kDa P-subunit. The P-subunit is the ubiquitous endoplasmatic reticulum (ER) resident enzyme PDI and its disulfide isomerase activity is not required to support the function of the MTP complex. The M-subunit requires the P-subunit for its solubility, its retention in the ER, and for lipid-transfer activity. The two subunits are held together by non-covalent interactions. (Hussain et al. 2003. *J. Lipid. Res.*)

The M-subunit consists of four structural elements (Figure 1.2.A) and the following three functional domains (Figure 1.2.B):

- lipid transfer domain
- apoB binding domain
- membrane association domain



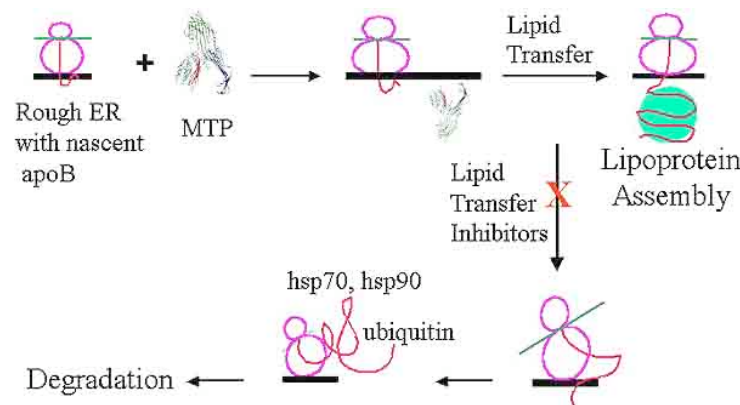
**Figure 1.2. Structural and functional domains of the M-subunit of MTP.**

A. Structural domains: the M-subunit consists of 3  $\beta$ -sheets and 1 helical domain. B. Functional domains: apoB binding, lipid transfer, and membrane association domains. (Hussain et al. 2003. *Front. Biosci.*)

---

#### 1.2.1.1. The lipid transfer domain

The lipid transfer domain plays an essential role in the assembly of lipoproteins by two independent mechanisms. First, MTP can take up lipids from the ER membrane and transfer them to nascent apoB. Several rounds of transfer would lead to the formation of a primordial lipoprotein particle. Insufficiently lipidated apoB is recognized by heat shock proteins, ubiquitinated, and targeted to degradation. Second, the lipid transfer domain might be involved in the formation and stabilization of lipid droplets, effecting the formation of lipoprotein particles. (Figure 1.3) (Hussain et al. 2003. *Front. Biosci.*)

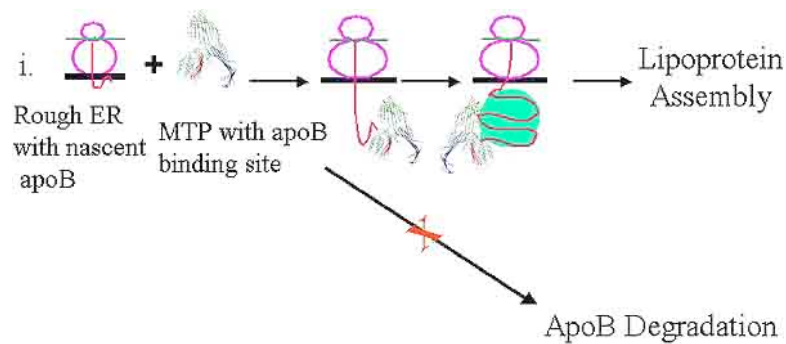


**Figure 1.3. Lipid transfer activity.**

Nascent apoB is associated with the ER membrane. MTP, bound to the ER membrane, picks up lipids from the membrane and transfers them to apoB. Inhibition of lipid transfer leads to ubiquitinylation and heat-shock protein binding enhancing the degradation of apoB. (Hussain et al. 2003. *Front. Biosci.*)

#### 1.2.1.2. The apoB binding domain

The N-terminus of apoB is exposed to the luminal side of the ER. Through binding of MTP to this N-terminus, it could release nascent apoB from the ER membrane liberating apoB's hydrophobic binding domains and making them available for lipidation. Further, MTP binding to apoB could promote specific transfer of lipids to nascent apoB instead of futile transfer of lipids between membranes. After extensive lipidation and formation of an intermediate lipoprotein, MTP will be released, leading to formation of a primordial lipoprotein particle. Inhibition of the apoB-MTP interaction may increase the degradation of apoB. (Figure 1.4) (Hussain et al. 2003. *Front. Biosci.*)

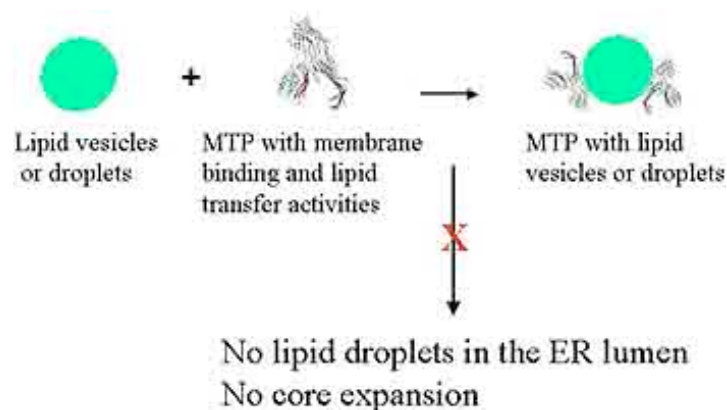


**Figure 1.4. ApoB binding activity.**

MTP interacts with nascent apoB leading to partial dislocation of apoB from the ER membrane. This provides a hydrophobic region in apoB for MTP to deliver lipids. Inhibition of apoB-MTP interaction could result in apoB degradation. (Hussain et al. 2003. *Front. Biosci.*)

#### 1.2.1.3. The membrane association domain

The membrane association domain may be involved in the formation and stabilization of lipid droplets. MTP bound to these droplets could advance the maturation of lipoprotein assembly by delivering lipids as a bolus. Lipid-associated MTP has a higher affinity for apoB and therefore promotes the biogenesis of primordial lipoprotein particles. Inhibition of the membrane association domain of MTP may decrease lipid droplet formation in the lumen of the ER and therefore impairs core expansion of the primordial particles. (Figure 1.5) (Hussain et al. 2003. *Front. Biosci.*)



**Figure 1.5. Membrane binding activity.**

MTP molecules associate with lipid droplets and stabilize them favoring lipid transfer. Inhibition of this interaction may decrease the number of lipid droplets in the ER lumen and further impairs core expansion of primordial particles. (Hussain et al. 2003. *Front. Biosci.*)

---

### 1.2.3. Functions of MTP

The major functions of MTP are its chaperone and lipid transfer activities during the formation of apoB-containing lipoproteins, as previously described. In this context, it is interesting to note that MTP has binding sites and lipid transfer activity for both phospholipids and neutral lipids, such as triglycerides and cholesterol esters. (Shelness and Ledford. 2005)

Beside its role in apoB lipoprotein assembly, MTP is also involved in CD1d (cluster of differentiation 1d) biosynthesis. CD1d is a major histocompatibility complex class I homolog that presents lipid and glycolipid antigens to natural killer (NK) T cells, which are able to rapidly produce cytokines early in an immune response. This molecule has a hydrophobic cleft for harboring and presenting lipid antigens and it is present on the surface of antigen presenting cells. CD1d is synthesized in the ER, where it associates with membrane lipids. Later on, these lipids are exchanged with antigenic self lipids or lipids of microbial origin.

Recent findings showed that CD1d physically interacts with MTP and the inhibition of MTP decreases cell surface expression of CD1d. Therefore, lipid antigen presentation is inhibited and NK T cell mediated immune response does not occur. In the ER, MTP directly transfers phospholipids to the antigen-binding site of CD1d and acts as a chaperone as it does for apoB-lipoprotein assembly. (Hussain et al. 2008)

### 1.2.4. Regulation of MTP

To characterize the molecular mechanisms controlling MTP gene transcription, human and hamster MTP gene promoters were analyzed. MTP transcription is initiated at a so called initiator sequence 30 basepairs (bp) downstream of the TATA box. There is a high sequence conservation 200 bp 5' to the transcription start site leading to the suggestion that important regulatory elements are present therein.

Both, human and hamster MTP promoters contain consensus recognition sequences for hepatocyte-specific trans-factors HNF-1 and HNF-4, an AP-1 recognition sequence, and a negative response element for insulin. HNF-4 is an activator of genes involved in lipid metabolism such as apoA-I, apoC-III, and apoB, and it is a member of the steroid

---

hormone receptor superfamily of transcription factors. Some members of this superfamily respond to fatty acids. The sterol regulatory element (SRE) in the human and hamster promoters was shown to slightly upregulate MTP expression by sterols. In contrast, insulin negatively regulates MTP gene transcription. (Hagan et al. 1994)

It has been further investigated, whether MTP mRNA levels are influenced by diet. Hamsters were fed different diets, leading to the finding, that a high fat diet increases mRNA and protein levels of MTP in intestine and liver, accompanied by significant changes in plasma cholesterol and plasma triglyceride levels. (Lin et al. 1994)

### 1.3. MTP in humans

In humans, apoB and MTP are involved in the secretion of lipoproteins by the liver (apoB100) and the intestine (apoB48). MTP's function is the transfer of neutral lipids onto nascent apoB leading to the formation of a lipoprotein particle for secretion. These lipoproteins enable the supply of peripheral organs with energy, fat-soluble vitamins and essential fatty acids. (Shelton et al. 2000)

#### 1.3.1. Abetalipoproteinemia

Genetic deficiency of MTP results in the autosomal recessive disease of abetalipoproteinemia. It is caused by mutations in the gene for the 97 kDa M-subunit of MTP. Most of the mutations found in the MTP gene of abetalipoproteinemia patients lead to truncated proteins devoid of function, but also some missense mutations are known that cause a milder form of the disease. In patients suffering from abetalipoproteinemia, apoB is synthesized but cannot form lipoproteins and therefore is degraded. Defects involving apoB lead to homozygous hypobetalipoproteinemia, a very similar condition. (Raabe et al. 1998; Tarugi et al. 2007)

In case of abetalipoproteinemia, the absorption and peripheral delivery of vitamins A and E are impaired, causing the pathophysiology of this inherited disease. Therapeutic



---

supplementation of these vitamins improves or even prevents complications when therapy started early in the course of the disease (Shelton et al. 2000). The plasma lipid profile of abetalipoproteinemia patients is characterized by extremely low plasma levels of total cholesterol, VLDL, and LDL, and an almost complete absence of apoB (Tarugi et al. 2007). In addition, fat accumulations in the intestine and hepatic steatosis have been observed in affected patients (Hussain and Bakillah. 2008).

### 1.3.2. MTP as a target in hyperlipidemias

As MTP is a chaperone for the assembly of lipoproteins, it seemed conceivable that inhibiting MTP activity might result in lower plasma cholesterol levels. Hyperlipidemias are risk factors of various cardiovascular and metabolic disorders, caused by reduced catabolism or increased production of lipoproteins. Statins reduce plasma cholesterol, but also cause adverse coronary events. Therefore, the development of new drugs is necessary. MTP, which is crucial for the synthesis of lipoproteins, seems to be an ideal candidate target of new drugs. Several MTP antagonists have been shown to inhibit triglyceride transfer activity in vitro, to suppress lipoprotein assembly and secretion in vivo, and to decrease plasma lipid levels in humans and animals. Major side effects of MTP inhibition first appear in intestine and liver in form of steatorrhea and diarrhea due to lack of chylomicron assembly by enterocytes, as well as upregulation of liver enzymes specific for liver damage. In liver, MTP inhibition also causes a higher accumulation of neutral lipids, which might be toxic. (Hussain and Bakillah. 2008)

To lower cellular cholesterol levels, inhibition of hepatic 3-hydroxy-3-methyl-glutaryl (HMG) CoA reductase might be helpful. There are also many other approaches to avoid side effects of MTP inhibitors to ensure their safety and efficacy, such as liver X receptor (LXR) agonists, which would enhance the efflux of free cholesterol. The development of such drugs could be very useful in the treatment of various lipidemias and metabolic disorders. (Hussain and Bakillah. 2008)

Extensive in vitro studies with cultured cells and MTP inhibitors showed that MTP activity does not appear to be limiting for the synthesis and assembly of apoB-containing

---

lipoproteins. Rather, the availability of lipids (triglycerides) limits the production of VLDL and chylomicrons. (Dixon et al. 1990; Pan et al. 2001)

### 1.3.3. MTP during human embryonic development

Embryos affected with abetalipoproteinemia or homozygous hypobetalipoproteinemia do not lead to fetal loss in humans, whereas homozygous apoB or MTP knockout mouse embryos are not viable. This could be due to the different function of the yolk sac in humans and rodents. In humans, the yolk sac seems to be only a vestigial remnant, whereas in rodents it has important nourishing functions during the critical phase before the establishment of placental, and it expresses the apoB gene and synthesizes apoB-containing lipoproteins. ApoB is also expressed in the human yolk sac, but as it is only a rudimentary organ, it loses its nutritional function very early in embryogenesis when the placenta is established. (Madsen et al. 2004)

In humans, the placenta, responsible for the full supply of the embryo with nutrients, expresses apoB and MTP. It was shown that it also synthesizes and secretes apoB100-containing lipoproteins. Formation and secretion of apoB lipoproteins is the most efficient way for delivery of lipids and carrying all the included nutrients such as essential lipids from the liver and intestine to peripheral organs or from the maternal circulation to that of the embryo. Beside liver and intestine, the placenta could be added to the list of apoB producing organs. ApoB is also expressed in heart myocytes. In mouse, apoB mRNA was also detected in kidney (Madsen et al. 2004). As the kidney of the chicken plays also a major role in lipoprotein production it seems plausible that it expresses MTP too (Ivessa et al. in revision).

---

## 1.4. MTP during mouse embryonic development

MTP is essential for the formation of apoB lipoproteins. ApoB knockout mice showed exencephalus and death in midgestation mouse embryos. Obviously, apoB-lipoprotein assembly in the developing embryonic yolk sac is required for development of the mouse embryo. (Gordon et al. 2000)

Before the establishment of the maternal-fetal circulation, apoB-lipoproteins could serve as a means of transport of essential lipids and lipid-soluble vitamins. As MTP is required for the formation of these lipoproteins, it has been suggested that knockout of MTP leads to the same phenotype. In fact, most of the homozygous MTP knockout mouse embryos died around embryonic day 14 and those who remained viable showed exencephalus (Raabe et al. 1998). These embryos showed severe neurodevelopmental defects and a lack of hematopoiesis, a phenotype very similar to apoB homozygous knockout mice (Gordon et al. 2000). Obviously, embryonic apoB lipoprotein assembly is critical for normal embryonic development of the nervous and the hematopoietic system in mice.

During the early stages of embryonic development, before establishment of the placental circulation, the yolk sac of rodents and other small mammals exposes its absorptive surface towards the uterine decidual tissue absorbing maternal nutrients including lipoproteins. These lipoproteins must be unloaded on the apical side of the yolk sac to enable their repackaging and re-secretion on the embryonic side of the membrane. Supply with nutrients by the yolk sac is especially important before the neural tube closes and before a functional circulatory system is established in the growing embryo. The uptake of lipoproteins by the yolk sac requires a set of lipoprotein receptors, including the low density lipoprotein (LDL) receptor gene family, as well as the scavenger receptor class B type I (SR-BI), a high density lipoprotein (HDL) receptor. Within the yolk sac, apoB and MTP mediate the repackaging of the lipids into lipoproteins (Herz and Farese 1999). Without apoB lipoprotein assembly, the supply of the embryo with lipids and lipid-soluble vitamins would not be possible. It was shown, that in the yolk sac of homozygous

---

apoB or MTP knockout mice lipids accumulated in large vacuoles within the yolk sac and are not transported to the growing embryo (Gordon et al. 2000).

Expression of MTP was also detected in liver and intestine of adult mice. In the liver, MTP was expressed at a higher level in the hepatocytes around the portal vein, and in intestine the expression was restricted to the enterocytes lining the villi.

In mouse embryos, MTP is first expressed in the yolk sac, followed by the primordial liver and subsequently in the developing intestinal tract. Further, the mouse yolk sac endoderm was characterized as a lipoprotein-secreting organ. (Terasawa et al. 1999)

### 1.5. Chicken MTP

Chicken MTP consists of 893 aa, it has a typical amino-terminal signal sequence with a predicted cleavage site between aa 19 and 20 and two consensus sites for N-linked glycosylation at aa 175 and 551. Compared to the chicken 97 kDa M-subunit with 893 aa, those of human, bovine, and murine consist of 894 aa residues and the one of hamster contains even 895 aa. Sequence homology analyses of chicken and human MTP showed that the two most conserved regions are located between aa 519 to 576 and 663 to 734 whereas the most N- and C- terminal regions are less conserved. (Ivessa et al. in revision)

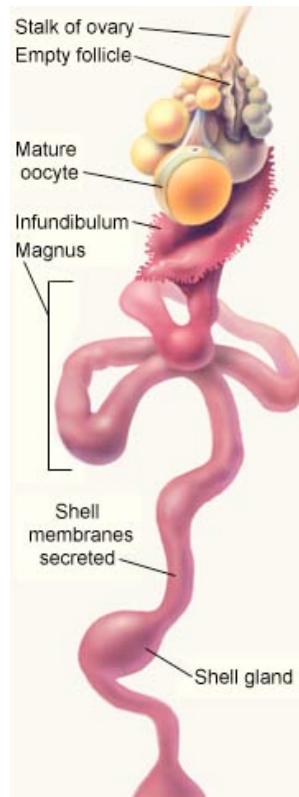
MTP expression analyses in the laying hen showed, that the expression level of the protein is much higher in small intestine than in liver and kidney. It was also shown, that estrogen-treatment of roosters had no influence on the expression level of MTP. Further in vitro cell culture experiments using the estrogen-responsive leghorn male hepatoma (LMH)-2A cell line also confirmed that MTP expression is not influenced by estrogen or derivatives. (Ivessa et al. in revision)

---

## 1.6. Embryonic development of the chicken

### 1.6.1. Oocyte formation and fertilization

Before embryonic development can start, the oocyte has to mature. The oocyte is a single massive cell filled with lipid-rich droplets and proteinaceous granules, the cytoplasm being restricted to the germinal disc, which is located at one pole of the cell. Maturation of the oocyte occurs in the ovary of the laying hen, where lipids, including VLDL and Vtg, synthesized by the maternal liver, are incorporated into the growing cell. After the synthesis in the liver, VLDL (apoB, apoVLDL-II) and Vtg are transported via the bloodstream to the oocyte and are easily taken up by receptor-mediated endocytosis. After incorporation into the oocyte, the yolk precursors VLDL and Vtg are proteolytically modified. ApoB is cleaved into a series of smaller polypeptides, whereas Vtg is cleaved at a specific site, giving rise to two proteins, phosvitin and lipovitellin. After ovulation, which takes place every 25 hours, the ovum is released from the ovary and enters the oviduct. Movement of the ovum down the oviduct is driven by peristaltic contractions. Fertilization occurs in the upper part of the oviduct, the infundibulum, followed by sequential addition of albumen proteins, shell membranes and shell formation in the lower part (Figure 1.6). (Speak et al. 1998; Moran 2007)



**Figure 1.6. Ovary and oviduct of the laying hen**

(chickscope.beckman.uiuc.edu.jpg)

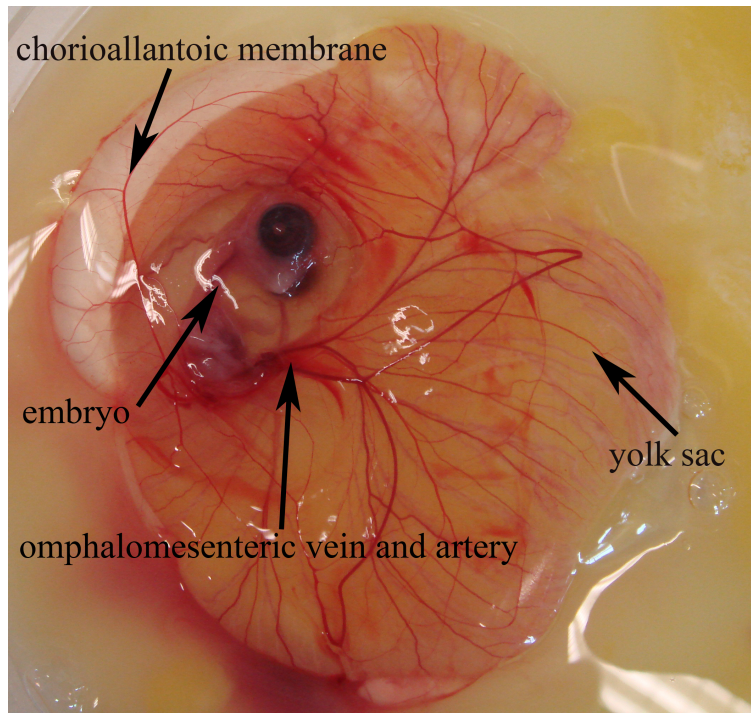
### 1.6.2. Early embryonic development

At the time of laying, the blastodisc with a diameter of about 4 mm is formed and floats on the surface of the yolk mass. Lipids, proteins, vitamins, and trace elements are pre-packaged in the egg and are required for the formation and growth of the embryo.

Extraembryonic tissue, including amniotic, chorioallantoic, and yolk sac membranes, are formed during the early stages of embryonic development, because their functions are essential for growth (Figure 1.7).

The chorioallantoic membrane lines the inner surface of the shell membranes and mediates the exchange of  $O_2$  and  $CO_2$  by its dense blood capillary system. Further, it mobilizes  $Ca^{2+}$  of the shell to fulfill the embryo's needs for bone formation.

The yolk sac membrane is also characterized by a high vascularization, and it grows outward from the embryo engulfing the yolk. Its function is the uptake of lipids from the yolk, their processing and transfer to the embryonic circulation. (Speak et al. 1998)



**Figure 1.7. 10-day-old chicken embryo.**

The embryo is connected to the yolk sac via the omphalomesenteric vein and artery. The yolk sac engulfs the yolk, providing the embryo with nutrients. The chorioallantoic membrane is pervaded with a dense blood capillary system for the exchange of  $O_2$  and  $CO_2$ .

#### 1.6.4. The yolk sac

The yolk sac membrane can be divided into two layers: the outer mesoderm, which performs a supportive role and the inner endoderm of columnar cells, responsible for yolk absorption. The apical surface of these endodermal epithelial cells (EECs) represents the absorptive surface of the yolk sac. To enable sufficient uptake of nutrients and supply of the embryo with them, these cells form folds, invaginations, and villus-like projections into the yolk. In addition, the EECs themselves form microvilli to enlarge the absorptive surface. The yolk sac is also pervaded by a network of capillaries, which emanate from the villi and feed into the major omphalomesenteric veins, which enter the portal circulation of the embryo through the yolk stalk (Figure 1.7).

---

Electron microscopy studies of the yolk sac gave some insights into the process of lipid uptake by the EECs. Lipid droplets and proteinaceous granules of the yolk are engulfed or phagocytosed at the apical surface of the EECs, or they are taken up in coated-pit regions via receptor-mediated endocytosis. It has been observed that inside the cells, the vesicles form large apical vacuoles. They fuse and give rise to large lipid-rich yolk droplets, which occupy the bulk of the cytoplasmic space. Inside these droplets, lipids are hydrolyzed and further processed.

Furthermore, the yolk-derived lipids are re-esterified in the ER and the Golgi, illustrated by the presence of lipid-rich spherules. This re-esterification may be intimately connected with lipoprotein assembly. The formation of lipoproteins requires apoB, the major structural protein of chylomicrons and VLDL, which associates with newly synthesized neutral lipids in the lumen of the ER, a process that requires MTP.

Finally, after processing the lipids are transported to the basal membrane of the EECs via exocytotic vesicles, which fuse with the cytoplasmic membrane and release the contents of the spherules into the vascular system of the yolk sac. (Speak et al. 1998)

#### 1.6.5. The liver

The omphalomesenteric veins feed into the portal system. Yolk sac-derived VLDL enters the embryonic circulation and primarily supplies the liver. As the lipoprotein lipase (LPL)-activity of embryonic liver is very low, most of the lipoproteins emerge from this tissue with little or no modification. VLDL catabolism is initiated in capillaries, which permeate the adipose tissue, heart and muscle. In these tissues LPL is more active. The remnant lipoproteins are removed from the bloodstream by receptor-mediated uptake into the liver, leading to massive accumulation of cholesterol esters (CE) in the hepatocytes, appearing as large lipid droplets in the cytoplasm. During embryonic development, continuous accumulation of CE in the liver occurs. Overspill of CE from the liver passes into the small intestine and finally ends up in the yolk sac. Thus, the yolk sac changes color, from yellow to green, towards the end of embryonic development.

It seems only logical that the lipid components of the remnants also undergo lysosomal hydrolysis and re-esterification in the ER of the embryonic liver, similar to the



---

modification of yolk-derived lipids by the yolk sac. This suggestion is supported not only by the presence of endocytic vesicles in the embryonic liver, but also by the expression of apoB mRNA in this organ. In addition, it has been shown by incubation of embryonic liver with labeled amino acids, followed by immunological detection that it synthesizes and secretes apoB. (Speak et al. 1998)

#### 1.6.6. The gastrointestinal system

The gastrointestinal system is not yet completely developed and functional in the chicken embryo. The embryo orally takes up the albumen-amniotic mixture, which is partially absorbed by enterocytes in the duodenum and jejunum. These enterocytes of the embryonic villi are capable of fluid and macromolecular transfer to supply the growing vascular system with albumen proteins. (Moran. 2007)

#### 1.6.7. The kidney

In the chicken, the kidney is a major lipoprotein-producing organ. The proximal tubule epithelium of the kidney expresses also receptors for uptake of various ligands, including apolipoproteins. This direct uptake of lipids in the proximal tubules may indirectly affect plasma and tissue lipid metabolism. It was shown that male chick kidney and rooster kidney express apoB RNA and synthesize apoB100. Further, it is known that laying hen kidney also expresses MTP, which is necessary for apoB-synthesis. Formation of apoB lipoproteins is an evolutionarily conserved system to effectively remove bulk lipids from cells. Therefore, apoB secretion could be a way for assembly and re-secretion of lipids from renal proximal tubule epithelial cells to the circulation in form of lipoproteins. (Ivessa et al. in revision; Moestrup et al. 2005; Walzem et al. 1999)

#### 1.6.8. Late embryonic development and hatching

The days before hatching, the yolk sac is completely retracted into the body of the embryo. So the newly hatched chick has about 5 g of residual yolk serving as nutrient

---

supply for the first days of its life. First food ingestion does not occur till the third day after hatching, and by day five after hatching, the remnant yolk sac is just a vestigial structure. (Speak et al. 1998)

In contrast to the yolk sac, which degenerates, the liver can get rid of its massive accumulation of CE by production and secretion of apoB- and apoAI-containing lipoproteins. Further, it starts to express lipolytic enzymes for the recycling of ingested food lipid components. (Speak et al. 1998; Tarugi et al. 1994)

Regarding the gastrointestinal system, enterocytes are arising from the crypts of the intestine and provide competence for digestion and absorption of nutrients. Within two weeks after hatching, the whole intestine is fully functional. (Moran. 2007)

### 1.7. The role of EECs

At the level of lipid content and fatty acid composition, distinct differences between the yolk contents and the embryonic plasma can be observed, and there are also differences between the structures and lipid contents of the major lipoprotein classes. (Noble and Cocchi. 1990)

The occurrence of resynthesis and reassembly of VLDL in the yolk sac has been emphasized by biochemical analyses of yolk VLDL and embryonic VLDL. Yolk VLDL contains cathepsin D-generated apoB fragments and apoVLDL-II, while embryonic VLDL contains apoB100 and lacks apoVLDL-II. (Hermann et al. 2000)

ApoB100 and apoVLDL-II are the two major apolipoproteins contained in VLDL in the hen, which are taken up into the growing oocyte via receptor-mediated endocytosis. ApoB100 mediates receptor binding, whereas apoVLDL-II is a lipoprotein lipase inhibitor and therefore assures full delivery of VLDL to the yolk. In the oocyte, apoB100 is proteolytically cleaved and stored as so called yolk VLDL. (Hermann et al. 2000)

The EECs, lining the inner surface of the yolk sac, are supposed to take up VLDL particles from the yolk via receptor-mediated endocytosis. Uptake studies with

---

fluorescently labeled yolk VLDL showed that cultured EECs actively take up lipoproteins. VLDL synthesis within the yolk sac requires MTP, which is also expressed therein. Further, apoB100 containing VLDL particles are present in the omphalomesenteric vein, which is part of the embryonic circulation. The lack of apoVLDL-II therein makes these lipoproteins substrates for embryonic lipoprotein lipase, allowing the embryo to derive energy from the yolk. (Hermann et al. 2000)

Taken together, these findings confirm the resynthesis and reassembly of yolk VLDL in the yolk sac and the important role of EECs in the uptake and processing of lipoproteins. It only remains to be demonstrated the secretion of newly synthesized apoB100 lipoproteins by these cells.

## 1.8. Aim of the thesis

During my diploma thesis, I wanted to investigate the role of MTP in embryonic and extraembryonic tissues. As the growing chicken embryo depends on the yolk as the unique source of energy, it nourishes on the lipids present therein. Findings about MTP, crucial for lipoprotein synthesis during this important period of life, could give further insights in its role in lipoprotein synthesis. Yolk sac, liver, small intestine, and kidney were the principal tissues to be analyzed with various methods to gain insights into the expression profile of MTP protein and mRNA, as well as its localization in various tissues, and its role in de novo synthesis of apoB-containing lipoproteins by the yolk sac.

---

## 2. Materials and Methods

### 2.1. Chemicals and enzymes

Chemicals obtained from AppliChem, Fluka, Merck, Riedel-de Haen, Roche, Sigma-Aldrich and Starlab were used for the production of buffers, solutions, and media.

The following polymerases were used:

SUPERScript<sup>TM</sup> RNase H<sup>-</sup> Reverse Transcriptase II (200 U/  $\mu$ l) from Invitrogen  
High Fidelity Enzyme Mix (5 U/  $\mu$ l) from Fermentas

### 2.2. Bacterial strains and vector systems

Strain	One Shot TOP10 Chemically Competent <i>E.coli</i>
Genotype	F <sup>-</sup> <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1</i> <i>araD139</i> $\Delta$ ( <i>araleu</i> )7697 <i>galU galK rpsL</i> (Str <sup>R</sup> ) <i>endA1 nupG</i>
Reference/ source	Invitrogen

**Table 2.1. Properties of chemically competent *E.coli* TOP10 bacteria**

Vector	pCR2.1-TOPO
Size	3931 bp
Genotype characteristics	LacZalpha fragment, M13 reverse priming site, MCS, T7 promoter/ priming site, M13 forward priming site, f1 origin, kanamycin resistance, ampicillin resistance, pUC origin
Reference/ source	Invitrogen, TA cloning kit

Table 2.2. Properties of T/A cloning vector

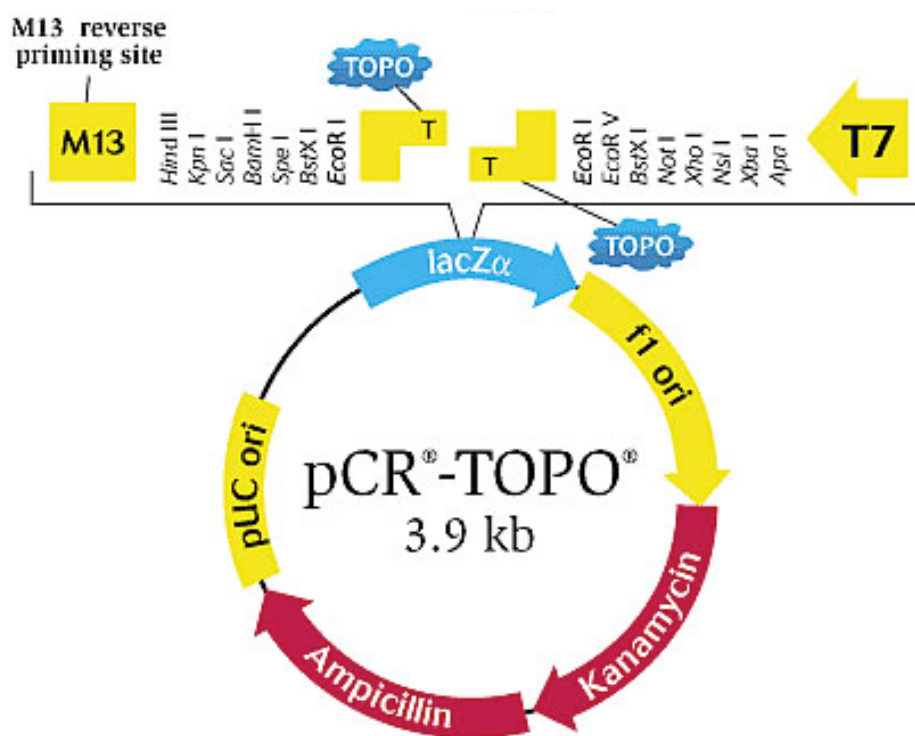


Figure 2.1. Genetic map of the pCR 2.1-TOPO cloning vector

([www.invitrogen.com](http://www.invitrogen.com))

---

## 2.3. Animals

Derco brown laying hens and roosters were purchased from Heindl Co. (Vienna, Austria). The animals were maintained on layer's mash with free access to water and feed under a daily light period of 16 hours. Fertilized eggs were incubated at 37,7°C and 55% relative humidity (from day 1 to day 19 of embryonic development) and 80% relative humidity (from day 20 until hatching) for a chosen time period.

## 2.4. Oligonucleotide primers

The designed oligonucleotide primers were synthesized by MWG-Biotech AG, Ebersberg, Germany.

Name	Sequence	Annealing°C
T/A cloning vector		
M13fw	5'- G TAA AAC GAC GGC CAG -3'	51.7
M13rev	5'- CAG GAA ACA GCT ATG AC -3'	50.4
Chicken MTP		
MTP_fw2	5'- GCTAGCCTTTTCCAGCTAC -3'	56.7
MTP_rev1	5'- ATTTTGGCACCTGTTTTTCG - 3'	54.0
Chicken ggRS17		
ggRS17_fw	5'- ACACCCGTCTGGGCAACGACT -3	63.7
ggRS17_rev	5'- CCCGCTGGATGCGCTTCATCA -3'	63.7
Standard primer		
Oligo (dT)	5'-TTT (TTT)2-4 TTT-3'	

**Table 2.3. List of oligonucleotide primers used for PCR**

---

## 2.5. Antibodies

Name	Epitope	Source
Anti-chicken MTP-B	peptide	Rabbit
Anti-chicken ApoB-100	protein	Rabbit
Anti-rabbit IgG peroxidase conjugated (Sigma)	Rabbit IgG	Goat
Anti-rabbit IgG biotin conjugated (Sigma)	Rabbit IgG	Goat

**Table 2.4. List of antibodies**

## 2.6. Molecular biological methods: DNA

### 2.6.1. cDNA synthesis

Total RNA was used as a template for the synthesis of cDNA, which was carried out using SUPERScript<sup>TM</sup> RNase H<sup>-</sup> Reverse Transcriptase II from Invitrogen. The following components were mixed together and incubated at 65°C for 5 minutes and subsequently put on ice:

10 µl RNA in RNase-free ddH<sub>2</sub>O

1 µl dNTPs (10 mM each)

1 µl Oligo(dT) (0.5 µg/ µl)

Afterwards, 4 µl First Strand Buffer and 2 µl 0.1M DTT were added followed by an incubation at 42°C for 2 minutes. Finally, 1 µl Superscript II reverse transcriptase (200 U/ µl) was added and mixed gently. The cDNA synthesis was performed at 42°C for 50 minutes and stopped by an incubation at 70°C for 15 minutes. Finally, the cDNA was stored at -80°C.

---

### 2.6.2. Polymerase chain reaction (PCR)

All components listed up below were mixed together in a sterile PCR-tube, which was kept on ice. Depending on the actual experiment, different primers were used. The PCR-program was also adapted depending on the best conditions for the current experiment.

cDNA	1 $\mu$ l
10 $\times$ High Fidelity PCR Buffer containing 15 mM MgCl <sub>2</sub> from Fermentas	2.5 $\mu$ l
Forward primer (20 pmol/ $\mu$ l)	0.5 $\mu$ l
Reverse primer (20 pmol/ $\mu$ l)	0.5 $\mu$ l
DMSO	1 $\mu$ l
dNTPs (10 mM each)	0.5 $\mu$ l
High Fidelity Enzyme Mix (5 U/ $\mu$ l) from Fermentas	0.25 $\mu$ l
ddH <sub>2</sub> O	18.75 $\mu$ l

**Table 2.5. Standard mixture of chemicals and enzymes used for PCR**

Step	Temperature	Time	Number of Cycles
Lid temperature	99°C		
Initial denaturation	94°C	5 min	
Denaturation	94°C	1 min	40
Annealing	x°C	x min	
Extension	72°C	x min	
Last Extension	72°C	10 min	
Pause	4°C		

**Table 2.6. Standard conditions used for PCR**



---

### 2.6.3. DNA gel electrophoresis

#### 50× TAE:

Tris/ HCl	2 M
Acetic acid	1 M
Ethylenediaminetetraacetic acid (EDTA)	0.1 M

#### Ethidium bromide stock solution:

10 mg/ ml in ddH<sub>2</sub>O

#### 5× DNA loading buffer:

100% Glycerine	5 ml
0.5 M EDTA	2 ml
50× TAE	12 ml
ddH <sub>2</sub> O	3 ml
Bromphenol blue or Xylene cyanol	

DNA fragments were separated by gel electrophoresis using a 1% agarose gel containing ethidium bromide (40 µg/ 100 ml) with a voltage of 100 V for 30 minutes. The samples were mixed with an adequate amount of 5× DNA loading buffer and loaded onto the gel together with 5 µl of GeneRuler™ 1 kb Plus DNA Ladder from Fermentas. The DNA fragments were visualized with ultraviolet light.

### 2.6.4. DNA gel extraction with Xact DNA gel extraction kit from GenXpress

The DNA-fragment was excised from the agarose gel with a sharp scalpel and put into an Eppendorf-tube. Then, 400 µl of Binding Buffer II were added to the gel. Further, the gel slice was incubated at 55°C for 10 minutes. During this incubation, the tube was vortexed several times. Thereafter, the mixture was added to the Xact column followed by incubation at room temperature for 2 minutes. The column was centrifuged for 2 minutes at 10.000 rpm in an Eppendorf centrifuge and the flow-through was discarded. The

---

membrane-bound DNA was washed twice by addition of 500 µl of Wash Solution, followed by centrifugation as described above. The column was centrifuged 2 minutes once more to remove any residual Wash Solution. Then, the column was placed in a clean 1.5 ml tube and 40 µl of pre-warmed (55°C) Elution Buffer were added to the center of the membrane. The column was incubated at room temperature for 2 minutes and finally the DNA was eluted by another centrifugation. The DNA was stored at -20°C.

#### 2.6.5. T/A-cloning

For the ligation, the TOPO TA Cloning® Kit from Invitrogen was used. 4 µl of purified DNA were mixed with 1 µl of salt solution and 1 µl of PCR 2.1 TOPO-vector followed by an incubation for 5 minutes at room temperature. 2 µl of the ligation reaction were used for the transformation.

#### 2.6.6. Transformation of competent *Escherichia coli* (*E.coli*) cells

##### Luria Broth plates containing kanamycin (LB-Kan plates):

Peptone from Casein	10 g
Yeast extract	5 g
NaCl	10 g
Agar-Agar	12 g
ddH <sub>2</sub> O	ad 1000 ml
Kanamycin	100 µg/ ml

##### LB-Kan plates containing X-Gal:

50 µl of X-Gal solution (50 mg/ ml) were plated on LB-Kan plates.

2 µl of the ligation were mixed with 50 µl of competent *E.coli* cells and put on ice for 30 minutes, followed by an incubation at 42°C for 30 seconds. Then, the mix was put on ice

---

again for 2 minutes. For regeneration of the *E.coli* cells 250 µl of SOC-medium from Invitrogen were added.

The mixture was incubated for 1 hour at 37°C with vigorous shaking. Finally, 50 µl and 150 µl were plated on LB-Kan/ X-Gal plates and incubated over night at 37°C. White colonies were analyzed for containing the correct insert by DNA mini preparation, restriction enzyme digestion, and agarose gel electrophoresis.

#### 2.6.7. Mini preparation of plasmid DNA

##### Luria Broth medium containing ampicillin (LB-Amp):

Trypton	10 g
Yeast extract	5 g
NaCl	10 g
ddH <sub>2</sub> O	ad 1000 ml
Ampicillin	100 µg/ ml

FastPlasmid™ Mini Kit from 5Prime was used for the preparation of plasmid DNA. All centrifugation steps were carried out in an Eppendorf microcentrifuge.

White *E.coli* colonies were picked from LB-Kan/ X-Gal plates and used for inoculation of 4 ml LB-Amp. 1.5 ml of this *E.coli* culture were centrifuged in an Eppendorf-tube at 14.000 rpm for 1 minute. Then, the medium was decanted and the pellet was resuspended in 400 µl ice-cold Complete Lysis Solution, followed by vortexing for 30 seconds. Afterwards, the lysate was incubated at room temperature for 3 minutes. After the incubation, the lysate was transferred to a Spin Column Assembly and centrifuged for 1 minute at 14.000 rpm. The column was washed once by adding 400 µl Wash Buffer and an additional centrifugation. Then, the column was centrifuged once more to dry it. Finally, the column was put into a Collection Tube and the DNA was eluted by adding 40 µl ddH<sub>2</sub>O and centrifuging 1 minute at full speed. The eluted DNA was stored at -20°C.

---

#### 2.6.8. DNA sequencing

For sequence analysis mini-preparations of plasmid DNA were sent to Agowa Genomics (Berlin, Germany).

#### 2.6.9. Quantitative real-time PCR

Quantitative real-time PCR was carried out using the Light Cycler® 480 system and the Light Cycler® FastStart DNA Master SYBR Green I Kit from Roche. First, the cDNA was diluted 1:10 with ultraPURE™ Distilled Water RNase, DNase free from GIBCO. Furthermore, a serial dilution from  $10^{-1}$  to  $10^{-9}$  of the purified PCR product was made which was used as internal standard.

Then, a mastermix was made and put together with the cDNA and the dilutions into a Light Cycler® Multiwell plate 96 from Roche.

ddH <sub>2</sub> O	4.5 µl
Forward Primer (20 pmol/ µl)	0.75 µl
Reverse Primer (20 pmol/ µl)	0.75 µl
Light Cycler® 480 SYBR Green I Master Mix	7.5 µl
cDNA/ purified PCR product	1.5 µl

**Table 2.7. Standard mixture of chemicals and enzymes for qPCR**

Step	Temperature	Time	Number of cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	15 sec	40
Annealing	68°C/ 67°C	15 sec	
Elongation	72°C	13 sec/ 10 sec	
Flourescence Measuring	79°C	3 sec	
Melting curve	95°C	10 sec	1
	65°C/ 75°C	20 sec	
	98°C*	1 sec	
Cooling	95°C	1 sec	1
	50°C	1 sec	

**Table 2.8. Standard program used for qPCR with chicken MTP primers.**

\*The temperature increases continuously from 65°C up to 98°C and the fluorescence intensity is measured 5 times per °C. *Italic:* Changes in time and temperature using chicken RS17 primers.

## 2.7. Molecular biological methods: RNA

### 2.7.1. Isolation of total RNA

Highly pure RNA for cDNA synthesis was isolated using the NucleoSpin® RNA II kit from Macherey-Nagel. All centrifugation steps were carried out in an Eppendorf microcentrifuge.

First, 35 mg of frozen tissue were homogenized in 350 µl buffer RA1 in a sterile glass potter. Thereafter, the homogenate was transferred into a 1.5 ml tube and 3.5 µl β-mercaptoethanol were added. The homogenized tissue was vortexed vigorously. The mixture was poured into a NucleoSpin filter unit, which was placed in a collection tube and centrifuged for 1 minute at 14.000 rpm. After this, 350 µl ethanol were added to the flow-through and the lysate was loaded onto a NucleoSpin RNA II column, which was placed in a 2 ml centrifuge tube. The mixture was centrifuged at 10.000 rpm for 1 minute.

---

Then, 350 µl Membrane Desalting Buffer were added to the column followed by another centrifugation at 14.000 rpm for 1 minute. In the meantime, 10 µl DNase I and 90 µl DNase reaction buffer were mixed and then added to the center of the membrane. After an incubation of 15 minutes at room temperature, 200 µl buffer RA2 were added to the column and the column was centrifuged at 10.000 rpm for 1 minute. After the first washing step, the membrane was washed another time with 600 µl buffer RA2 and a third time with 250 µl buffer RA3, whereas the centrifugation at the third washing step occurred at 14.000 rpm for 2 minutes. Finally, the column was placed in a nuclease-free Eppendorf-tube and the RNA was eluted with 40 µl RNase-free ddH<sub>2</sub>O by centrifugation at 14.000 rpm for 1 minute. After measuring the RNA-concentration the samples were stored at -80°C.

#### 2.7.2. Determination of RNA concentration

The RNA concentration was determined with the ND-100 Spectrophotometer from peqlab.

### 2.8. Molecular biological methods: protein

#### 2.8.1. Preparation of membrane protein extracts

##### Buffer A:

Tris/ HCl, pH 8            20 mM

CaCl<sub>2</sub>                      1 mM

NaCl                        150 mM

Complete, EDTA-free (Protease inhibitor cocktail tablets) from Roche

---

Buffer B:

Tris maleate, pH 6     250 mM

CaCl<sub>2</sub>                     2 mM

Complete, EDTA-free (Protease inhibitor cocktail tablets) from Roche

To 1 g of frozen or fresh tissues prepared from chicken embryos of different developmental stages or laying hens, 5 ml buffer A were added and the tissue was homogenized with polytron three times for 15 seconds. In the meantime, the homogenate was kept on ice as much as possible.

Thereafter, the homogenate was centrifuged at 5.000 rpm in the SS34 rotor from Sorvall in the Sorvall® RC 5C Plus centrifuge for 10 minutes. Then, the supernatant was transferred into a fresh tube and centrifuged again at 50.000 rpm in the TLA 100.3 rotor from Beckman in the Optima™ TLX Ultracentrifuge from Beckman for 1 hour. After this centrifugation, the supernatant was discarded and lipids were removed using a wipe.

Then, the pellet was resuspended in 3 ml of buffer A, using first a 18 gge needle, followed by a 22 gge needle. The suspension was centrifuged again at 50.000 rpm in the TLA 100.3 rotor from Beckman in the Optima™ TLX Ultracentrifuge from Beckman for 1 hour. After this, the supernatant was discarded again and the pellet was resuspended in 625 µl of buffer B using again the 18 gge and the 22 gge needles. Then, 50 µl 4M NaCl were added and the sample was sonicated for 30 seconds at 50% with the Bandelin Sonoplus HB 70 sonicator. Finally, 325 µl ddH<sub>2</sub>O and 250 µl 5% Triton X-100 were added followed by a final centrifugation at 50.000 rpm in the TLA 100.3 rotor from Beckman in the Optima™ TLX Ultracentrifuge from Beckman for 1 hour. Then, the supernatant was carefully aliquoted and stored at -80°C.

#### 2.8.2. Determination of protein concentration

Protein concentration of membrane protein extracts was determined with the method of Bradford using Assay Dye Reagent Concentrate from BioRad. 10 µg of BSA were mixed with 1 ml of the concentrate and used as standard. 1 µl of each membrane protein extract was also mixed with 1 ml concentrate. After 5 minutes of incubation at room temperature

---

the absorbance of the samples were measured with an UV spectrophotometer at 595 nm.  
The protein concentration was calculated as follows:

$$\frac{\mu\text{g Standard}}{\text{OD Standard}} \cdot \frac{\text{OD Sample}}{\mu\text{l Sample}} = \mu\text{g}/\mu\text{l Protein}$$

### 2.8.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

#### 1× Electrophoresis buffer:

Tris/ HCl	250 mM
Glycine	192 mM
SDS	0.1%

#### 4× Laemmli buffer (non reducing):

Glycerol	1.2%
SDS	6%
Tris/ HCl pH 7.5	20 mM
Bromphenolblue	
ddH <sub>2</sub> O ad 20 ml	

#### 4× Laemmli buffer (reducing):

Glycerol	1.2%
SDS	6%
Tris/ HCl pH 7.5	20 mM
Dithiothreitol (DTT)	50 mM
Bromphenolblue	
ddH <sub>2</sub> O ad 20 ml	



	Stacking gel	Separation gel		Gradient gel	
	4%	6%	10%	4.5%	18%
ddH <sub>2</sub> O (ml)	1.525	2.675	2.025	1.92	0.43
1.5 M Tris pH8.8 (ml)	-	1.25	1.25	0.81	0.81
0.5 M Tris pH 6.8 (ml)	0.625	-	-	-	-
30% PAA (ml)	0.325	1	1.65	0.495	1.98
10% SDS (μl)	25	50	50	32.25	31.5
10% APS (μl)	12.5	25	25	25	25
TEMED (μl)	5	5	5	2.5	2.5

**Table 2.9. Composition of SDS-gels**

SDS-PAGE was performed using the equipment from BioRad. All components of the separation gel were mixed and pipetted into the gel unit. In case of the gradient gel, ddH<sub>2</sub>O, Tris buffer, acrylamid, and 10% SDS were mixed and pipetted into the gradient maker. After addition of APS and TEMED, the two solutions were poured into the gel unit generating a gel ranging from 18% at the bottom to 4.5% at the top of the gel. The gel was overlaid with isopropanol, which was removed after polymerization of the gel. Then the stacking gel was mixed and poured onto the separation gel. The comb was inserted immediately. After polymerization of the stacking gel, the comb was removed and the slots were rinsed with ddH<sub>2</sub>O. Thereafter the gel was put into the buffer chamber, which was filled with electrophoresis buffer.

The samples were mixed with an adequate amount of reducing or non-reducing Laemmli buffer. Under reducing conditions, the samples were incubated for at least 5 minutes at 95°C, which is not necessary under non-reducing conditions. Then, the samples were loaded onto the gel together with 15 μl of Precision Plus Protein Standard from BioRad. The separation of the proteins occurred at a constant voltage of 130V for a time period depending on the size of the protein to be analyzed.

The gel was either used for Coomassie stain, Silver stain, or Western blot analysis.

---

#### 2.8.4. Coomassie stain

##### Coomassie blue:

Acetic acid	10%
Isopropanol	25%
Coomassie Brilliant Blue R250	0.287 g
ddH <sub>2</sub> O ad 1000 ml	

##### Destain solution:

Methanol	30%
Acetic acid	10%
Diluted with ddH <sub>2</sub> O	

SDS-gels were incubated in Coomassie Blue for 1 hour at room temperature. Then, Coomassie Blue was decanted and the gel was covered with Destain Solution for at least 1 hour at room temperature and further dried in a gel vacuum dryer.

#### 2.8.5. Western blot analysis

##### 1× Blotting Buffer:

Tris/ HCl	25 mM
Glycine	193 mM

##### 10× TBS:

NaCl	1.37 M
KCl	0.027 M
Tris/ HCl	0.25 M
With HCl to pH 7.4	

---

1× TBS-T:

1:10 dilution of 10× TBS

Tween-20      0.01%

Ponceau S:

Ponceau S              0.2%

Trichloroacetic acid    3%

Diluted with ddH<sub>2</sub>O

Blocking solution:

5% non-fat dry milk powder in 1× TBS-T

Detection solution:

Enhanced chemiluminescent (ECL) from Pierce

Wet blotting:

For wet blotting the blotting equipment from BioRad was used. Proteins were transferred from the SDS-gel to Hybond<sup>TM</sup>-C Extra for optimized protein transfer-membranes from Amersham Bioscience.

First, three Whatman papers were soaked with 1× Blotting Buffer and put onto the blotting unit. Then, the nitrocellulose membrane was applied to the Whatman papers, followed by the gel, and three more wet Whatman papers. To avoid air bubbles a Falcon tube was rolled over the stack. Then the blotting unit was connected to the power supply and blotting was performed for 1 and a half hour at 200 mA per gel.

After blotting, the nitrocellulose membrane was stained with Ponceau S. After destaining with ddH<sub>2</sub>O, the bands of the samples and of the standard were visible. The bands of the standard were marked with a pen.

Immunoblot:

The nitrocellulose membrane was blocked with 5% non-fat dry milk in 1× TBS-T for at least 1 hour at room temperature. Then, the primary antibody was diluted in 5% non-fat

---

dry milk in 1× TBS-T and the nitrocellulose membrane was incubated with it over night at 4°C gently shaking.

The next day, the nitrocellulose membrane was washed 3 times with 1× TBS-T for 5 minutes. Thereafter, the membrane was incubated with an HRP-conjugated secondary antibody diluted in 5% non-fat dry milk in 1× TBS-T for 1 hour at room temperature gently shaking. Then, the nitrocellulose membrane was washed another 3 times with 1× TBS-T for 5 minutes.

#### Detection:

Enhanced chemiluminescence (ECL) from Pierce was used to visualize the proteins on the nitrocellulose membrane. The peroxide solution and the luminal/ enhancer solution were mixed 1:1 and the nitrocellulose membrane was covered with this mixture and incubated for 2 minutes at room temperature. Thereafter, the membrane was put into an exposure cassette and CL-XPosure<sup>TM</sup> Films from Pierce were exposed to the signal in the dark room.

#### 2.8.6. Pulse chase

##### 1× PBS:

NaCl            137 mM

KCl             2.7 mM

Na<sub>2</sub>HPO<sub>4</sub>       10 mM

KH<sub>2</sub>PO<sub>4</sub>       2 mM

Diluted with ddH<sub>2</sub>O, pH 7.4

##### Starvation medium:

RPMI 1640 without L-Glutamine, L-Methionine, L-Cystine and L-Cysteine from MP Biochemicals

---

Pulse medium:

Starvation medium containing 250  $\mu\text{Ci/ ml}$  NEG-772 Easytag<sup>TM</sup> Express Protein Labeling Mix, [<sup>35</sup>S]

Chase medium:

DMEM with high glucose (4.5 g/ l), with D-Valine, without L-Valine, without L-Glutamine from PAA 500 ml

FCS 10%

AB/AM antibiotic, antimyotic from Gibco 5 ml

L-Glutamine (200 mM) 5 ml

L-Methionine (400 mM) 20  $\mu\text{l}$

L-Cysteine (400 mM) 20  $\mu\text{l}$

Protein A sepharose:

Protein A Sepharose<sup>TM</sup> CL-4B from GE Healthcare

Loading buffer:

4 $\times$  Laemmli buffer (reducing)

Cultured tissue-pieces and cells were washed twice with 1 $\times$  PBS and incubated in an appropriate amount of starvation medium for 30 minutes in an incubator (37°C and 5% CO<sub>2</sub>). Then, the medium was removed and the tissue-pieces and cells were exposed to the pulse medium for 1 hour in an incubator (37°C and 5% CO<sub>2</sub>). After the pulse phase, the specimens were covered with chase medium, which was retained after 5 minutes, 1, 2, and 4 hours.

---

#### 2.8.7. Immunoprecipitation

##### 10× PBS:

NaCl            1.37 M

KCl             27 mM

Na<sub>2</sub>HPO<sub>4</sub>       100 mM

KH<sub>2</sub>PO<sub>4</sub>       20 mM

Diluted with ddH<sub>2</sub>O, pH 7.4

##### 1× PBS:

1:10 dilution of 10× PBS with ddH<sub>2</sub>O

The samples (media from pulse chase analysis) were centrifuged for 15 minutes at 4°C and 15.000 rpm in the 1K15 centrifuge from Sigma. Then, the supernatant was collected and transferred to a new Eppendorf tube and mixed with 40 µl Protein A sepharose-beads and an appropriate amount of the antibody. The samples were rotated at 4°C over night.

The next day, the beads were washed 5 times by centrifuging 2 minutes at 8.000 rpm in an Eppendorf centrifuge, adding 1 ml 1× PBS and rotating 10 minutes at 4°C. Thereafter, the beads were mixed with loading buffer, the samples were put at 95°C for 5 minutes and cooled on ice.

#### 2.8.8. SDS-PAGE and detection

##### Fixing solution:

Acetic acid    10%

Methanol      30%

##### Enhancer:

EN3HANCE Autoradiography enhancer from Perkin Elmer

---

To collect the beads at the bottom of the tube, they were quickly spun down and the supernatants were loaded onto a SDS-gradient gel to separate the proteins. As a protein standard, NEC-811 protein molecular weight markers [methyl-<sup>14</sup>C] methylated from PerkinElmer was used.

After running the gel, it was incubated for 30 minutes in fixing solution, followed by another 30 minutes in enhancer gently shaking. Thereafter, the gel was rinsed 5 times with water and shaken for 25 minutes in water.

Then the gel was applied onto a Whatman paper and dried for 1 hour and 40 minutes at 80°C in a vacuum gel dryer. The gel was put into an exposure cassette and Amersham Hyperfilm<sup>TM</sup> MP films from GE Healthcare were exposed to the signal at -80°C for several days to weeks.

## 2.9. Histological methods

### 2.9.1. Paraffin sections

#### 4% paraformaldehyde (PFA):

PFA 4%

Dissolved in ddH<sub>2</sub>O at 55-60°C

pH 7.4

#### Blocking solution:

BSA 1%

Inactivated goat serum 3%

Dissolved in 1× PBS

Freshly isolated tissue was incubated over night in 4% PFA at 4°C. The next day, the tissue was washed twice with 1× PBS for 10 minutes. Then, it was incubated in 50%

---

ethanol at room temperature for 1 hour. After this, the tissue was put into embedding cassettes and into the embedding machine Shandon Excelsior over night.

Next day, the tissue was embedded in paraffin and stored at 4°C.

The embedded tissue was cut with the LeicaRM2155 microtome into sections of 8 µm and fixed on Polysine<sup>TM</sup> slides (Menzel-Glaeser). The sections were dried over night at 37°C or for 1 hour at 50°C. To deparaffinize the tissue the slides were incubated for 20 minutes in XEM-200 gently shaking followed by 100%, 90%, 70%, 50%, 30% ethanol 2 minutes each and finally 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes.

For Biotin-staining, the sections were incubated with blocking solution at room temperature for 1 hour. The primary antibody and the corresponding pre-immune serum were diluted with blocking solution and applied to the sections followed by an incubation at 4°C over night in a humid chamber.

Next day, the slides were washed three times for 10 minutes with 1× PBS. The secondary biotinylated goat-anti-rabbit antibody was diluted with blocking solution and the sections were incubated with it for 1 hour at room temperature. Then, the slides were washed another 3 times for 10 minutes with 1× PBS. Thereafter, the sections were covered with Streptavidin-HRP diluted with 1% BSA in 1× PBS and incubated at room temperature for 20 minutes. After three final washes of 10 minutes each with 1× PBS, the sections were covered with AEC+ Substrate-Chromogen, ready-to-use solution from Dako to get the color reaction. This color reaction was stopped with water, the slides were air-dried, mounted with Glycergel® Mounting Medium from Dako and stored at 4°C.

### 2.9.2. Cryosections

Frozen tissue was embedded in O.C.T.<sup>TM</sup> Compound Tissue-Tek® from Sakura at -24°C. Sections of 7 µm were cut with the HM500OM Microm and fixed on SuperFrost® Plus slides (Menzel-Glaeser). Then the slides were stored at -20°C.

For specific detection of proteins, the sections were put at room temperature for 10 minutes, followed by incubation in ice-cold acetone-methanol 1:1 gently shaking for 5



---

minutes. Thereafter, the slides were washed 3 times with 1× PBS for 5 minutes each. DakoCytomation Pen was used to draw a barrier to liquids. Then, the slides were incubated with the appropriate primary antibody diluted with 1× PBS over night at 4°C in a humid chamber.

Biotin:

After rinsing the slides 3 times 5 minutes each in 1× PBS, they were incubated with the secondary biotinylated goat-anti-rabbit antibody from Sigma diluted 1:500 with 1× PBS for 1 hour at room temperature. Subsequently, the slides were washed another 3 times 5 minutes in 1× PBS followed by incubation with Streptavidin Peroxidase Polymer from Sigma diluted 1:250 with 1× PBS. After three final washes of 5 minutes each with 1× PBS, the sections were covered with AEC+ Substrate-Chromogen, ready-to-use solution from Dako to get the color reaction. This color reaction was stopped with water, the slides were air-dried, mounted in Glycergel® Mounting Medium from Dako, stored at 4°C and analyzed by light microscopy.

---

## 2.10. Cell and Tissue Culture

### 2.10.1 Isolation of endodermal epithelial cells (EECs)

#### Hanks' balanced salt solution (HBSS):

CaCl <sub>2</sub> (anhydrous)	0.14 g/ l
KCl	0.40 g/ l
KH <sub>2</sub> PO <sub>4</sub>	0.06 g/ l
MgCl <sub>2</sub> •6H <sub>2</sub> O	0.10 g/ l
MgSO <sub>4</sub> •7H <sub>2</sub> O	0.10 g/ l
NaCl	8.00 g/ l
NaHCO <sub>3</sub>	0.35 g/ l
Na <sub>2</sub> HPO <sub>4</sub> •7H <sub>2</sub> O	0.09 g/ l
D-glucose	1.00 g/ l

The components were dissolved in ddH<sub>2</sub>O and the pH was adjusted to 6.0

#### Wash solution:

Horse serum 2%

In HBSS

#### Collagenase solution:

Collagenase 235 U/ ml

In wash solution

#### Medium:

DMEM with high glucose (4.5 g/ l), with D-Valine, without L-Valine, without L-

Glutamine from PAA 500 ml

FCS 10%

AB/AM antibiotic, antimyotic from Gibco 5 ml

L-Glutamine 200 mM 5 ml

Horse serum 2%

---

Endodermal epithelial cells were isolated from chick embryo yolk sacs of 8 to 10 days of incubation. First of all, the yolk sac was incubated in collagenase solution at 37°C for 4 hours. Thereafter, the yolk sac was cut into 5 mm pieces and incubated again in collagenase solution for 10 minutes at room temperature with intermittent shaking. Large pieces were removed with forceps and the solution was transferred to a 50 ml tube. Wash solution was added to a final volume of 50 ml and the tube was inverted a few times. During an incubation time of 20 minutes at room temperature, the younger cells sank to the bottom of the tube, whereas the more lipid-loaded older cells floated. These floating cells were removed together with the supernatant and the cells. The cells at the bottom were washed two more times by adding 50 ml wash solution, incubating the solution at room temperature for 20 minutes and carefully removing the supernatant. Then, the cells were resuspended in 15 ml wash buffer and filtered through a 100 µm nylon mesh. Endodermal epithelial cells remained on the filter and were released with culture medium, which was transferred to a fresh 50 ml tube, where the cells were allowed to settle again. Finally, cells were plated at high concentration and low volume onto collagen-coated culture slides or nylon mesh and incubated at 37°C in 5% CO<sub>2</sub>. After 24 hours a few drops of medium were added to each well and after 48 hours 0.5 ml of medium were added. Then, the medium was changed every other day.

#### 2.10.2. Cysteine lyase activity-assay

##### Reaction Solution:

Tris/ HCl pH 8.5	0.15 M
L-cysteine hydrochloride	10 mM
Sodium sulfite	10 mM
Lead acetate	1 mM
Pyridoxal 5-phosphate	10 µM

To verify the cultured cells being endodermal epithelial cells the cysteine lyase activity assay was performed.

---

First the cells were washed with 1× PBS. Then the cells were fixed for 5 minutes in 96% ethanol at 4°C, followed by a rinse in distilled water. Then the cells were incubated in reaction solution at 38°C for 90 minutes visualize cysteine lyase activity leading an insoluble brown precipitate.

### 2.10.3. Tissue culture

#### Medium:

DMEM with high glucose (4.5 g/ l), with D-Valine, without L-Valine, without L-Glutamine from PAA	500 ml
FCS	10%
AB/AM antibiotic, antimyotic from Gibco	5 ml
L-Glutamine 200 mM	5 ml

Yolk sacs were isolated from eggs of 15 to 18 days of incubation. Then, the yolk sacs were rinsed with HBSS to remove the yolk. Small pieces of 0.7 to 1 cm<sup>2</sup> were cut off and incubated in medium, which was changed every day.

After one week, most yolk was removed and the tissue pieces could be used for further experiments.

---

## 3. Results

### 3.1. Expression of MTP in chicken extraembryonic tissue

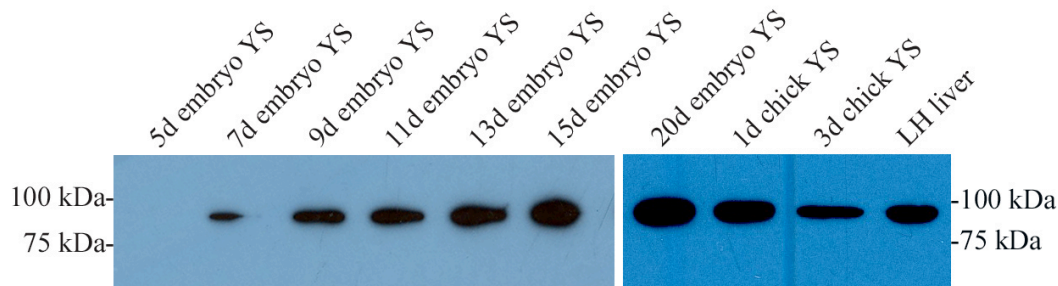
During my diploma thesis, the tissue of major interest was the chicken yolk sac. The yolk sac is one of the three extraembryonic tissues and is responsible for the supply of the embryo with nutrients from the yolk. It is supposed to take up lipids and re-synthesize them before their release into the embryonic blood system. As MTP is required for the synthesis of apoB-containing lipoproteins, I wanted to know, if and at which times it is expressed during embryonic development of the chicken.

#### 3.1.1. Western blot analysis

First, membrane protein extracts of yolk sacs of different developmental stages (day 5, 10, 15, and 20) were prepared, the proteins were separated by SDS-PAGE, and transferred to a nitrocellulose membrane. For the detection of MTP, an antibody, termed  $\alpha$ MTP-B, directed against the C-terminal aa 879 to 893 of the chicken pre-protein was used. (Described in detail in Materials and Methods, 2.8.5)

First, I determined that MTP expression in the yolk sac starts between day 5 and day 10 of embryonic development. To specify the point in time when MTP expression starts, and to visualize the supposed continual increase of MTP expression, further membrane protein extracts of intermediate developmental stages (day 7, 9, 11, and 13) were prepared. As the yolk sac is still present during the first days after hatching and also fulfilling a function, yolk sacs of 1- and 3-day-old chicks were included in my analyses.

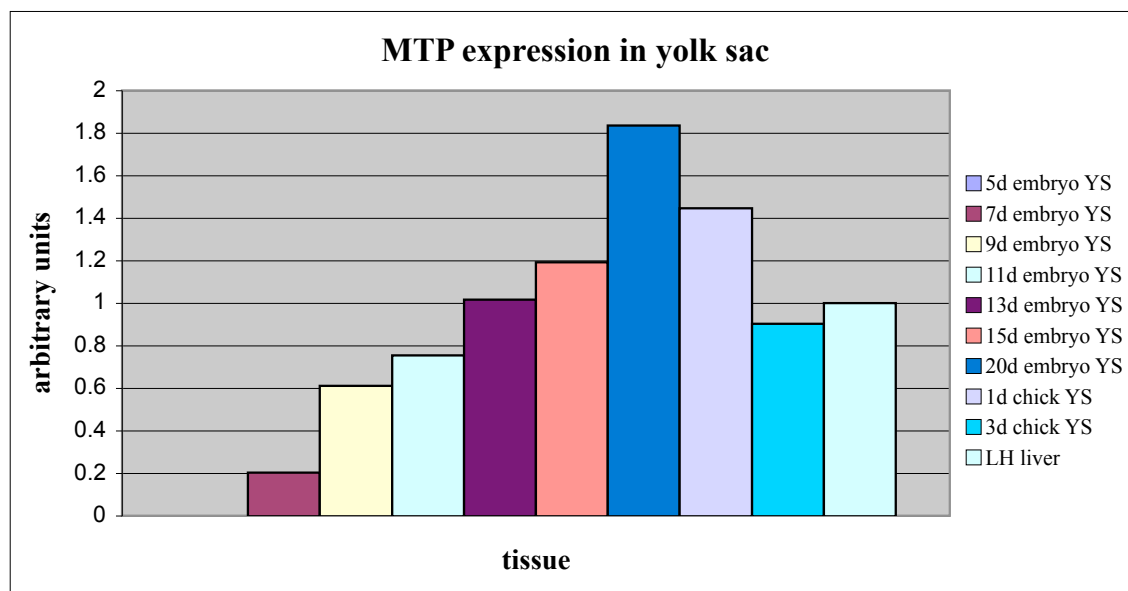
My results show that expression of MTP starts around day 7 of embryonic development. This is exactly after the first trimester, when the yolk sac starts to undertake the task of nourishing the embryo. Further, I could observe that MTP expression increases until day 20, one day before hatching and it is reduced thereafter, when the yolk sac still has a nourishing function, but already starts to shrivel. (Figure 3.1)



**Figure 3.1. Western blot analysis of MTP expression in the chicken yolk sac.**

10 µg protein of membrane protein extracts from yolk sacs of 5-day-old chicken embryos to 3-day-old chicks and from the liver of laying hen, used as a positive control, were used for 10% SDS-PAGE followed by western blot analysis. MTP was detected using the  $\alpha$ MTP-B antibody directed against a C-terminal peptide of the pre-protein, diluted 1:5000. The intensity of the band increases with age of the embryo. At day 20, the expression of MTP in the yolk sac peaks and it decreases rapidly after hatching.

Quantification with AlphaEaseFC led to more detailed information about the amount of MTP expressed in the yolk sac compared to the liver of laying hen, which served as a standard in western blots. The continuous increase of MTP expression towards day 20 of embryonic development and its decrease thereafter is clearly visible in the diagram (Figure 3.2). The decrease of the amount of detectable MTP after hatching could be due to degradation or a lower translation rate.



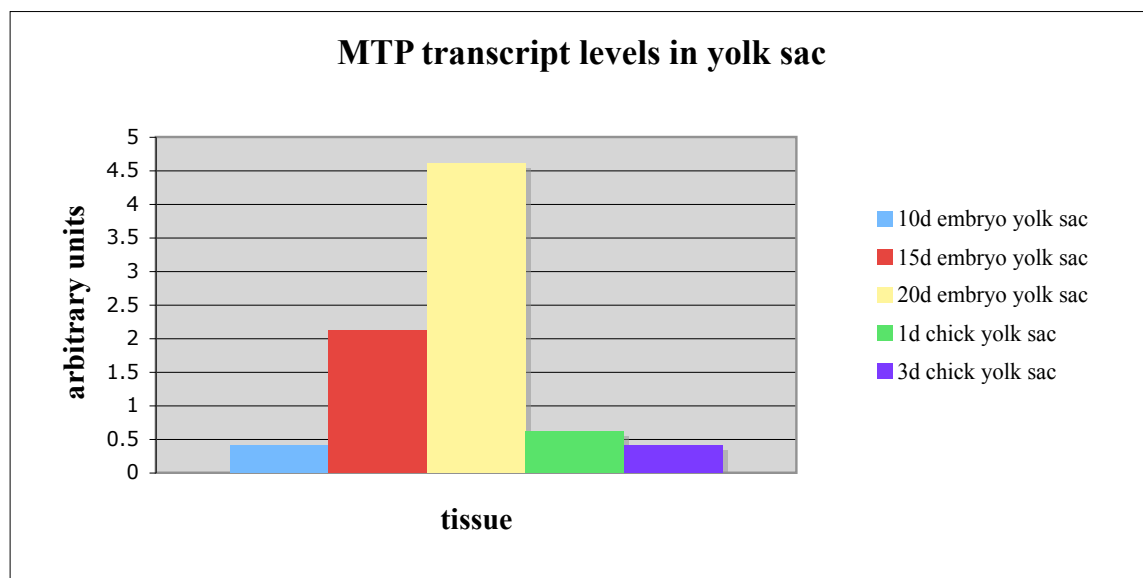
**Figure 3.2. Quantification of MTP expression in the yolk sac with AlphaEaseFC.**

At day 5 of embryonic development, MTP expression could not be detected, but from around day 7 on, it is present in the chicken yolk sac. The level of MTP expression massively increases resulting in a peak around day 20 (blue), showing an expression, twice as high as in the liver of the laying hen (pink).

### 3.1.2. Quantitative PCR

After analysis of MTP expression at the protein-level, I also wanted to analyse the transcript levels of MTP mRNA in the yolk sac. This was performed via quantitative PCR. After isolation of total RNA from yolk sacs of different developmental stages, cDNA was synthesized and used as a template for the quantitative PCR (for details see Materials and Methods 2.6.9).

At the mRNA level, similar results as at the protein level could be observed (Figure 3.3). The amount of MTP mRNA decreases considerably after hatching as well, indicating that MTP mRNA is no longer required in such huge amounts at this time. This could be due to a down-regulation of the MTP gene transcription.



**Figure 3.3. Course of MTP transcript levels in the yolk sac of chicken embryos and hatchlings.**

Similar to the protein level, the amount of transcripts peaks shortly before hatching, but decreases rapidly thereafter.

qPCR and western blot analysis confirmed the presence of MTP mRNA and protein in the yolk sac from the end of the first trimester of embryonic development on, suggesting that the amounts of mRNA as well as protein peak around day 20, shortly before hatching. The fact that most of the nutrients stored in the yolk are taken up during the third trimester supports the suggestion that most MTP, crucial for lipoprotein synthesis, is required during this period of development.

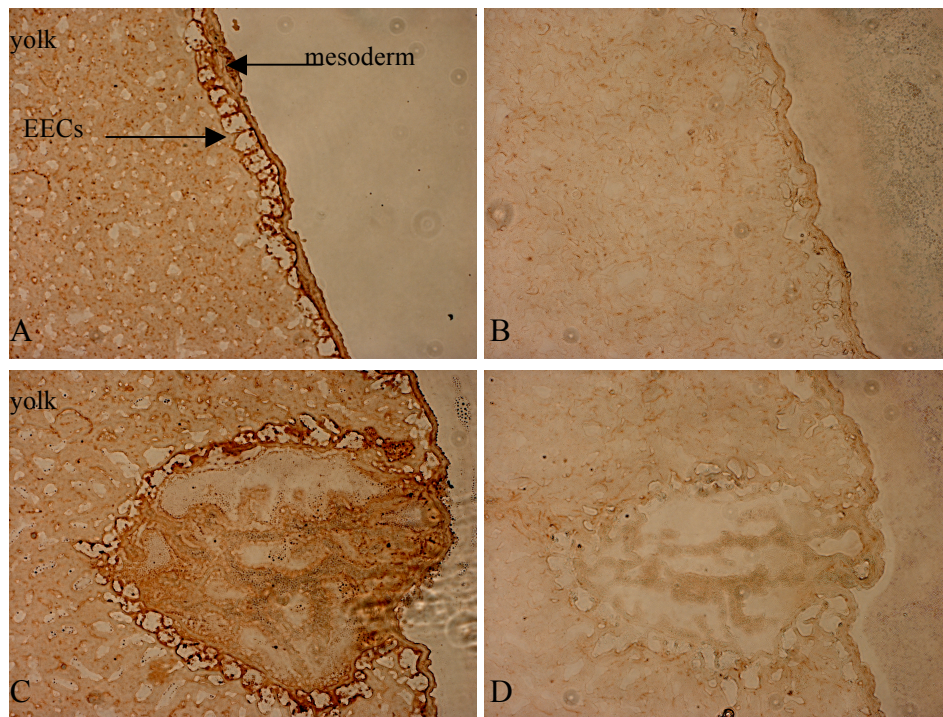
### 3.1.3. Immunohistochemistry

It may be assumed that the re-synthesis of lipoproteins occurs at the inner cell layer of the yolk sac, which faces the yolk and is constituted by EECs. As shown before by qPCR and western blot analysis, MTP is expressed in the yolk sac. To localize MTP in the yolk sac at an ultrastructural level I performed immunohistochemistry. Cryosections were the method of choice because of the possibility to freeze the whole yolk sac together with the yolk. Using this technique, I was able to generate sections showing the yolk sac membrane with its two cell layers (mesoderm and EECs) and the yolk (Figure 4.A).



---

Figure 3.4 shows two representative immunohistochemically stained cryosections from the yolk sac of a 15-day-old chicken embryo. A significant reddish-brown coloring indicating the expression of MTP is present in the yolk sac. According to the staining obtained, the EECs, directly facing the yolk, clearly express MTP, but also the outer layer of the yolk sac, the mesoderm, appears to have a slightly reddish-brown coloring. To clarify the question, whether MTP is indeed expressed in the EECs, and whether apoB de novo synthesis occurs therein, these cells were isolated from yolk sacs.



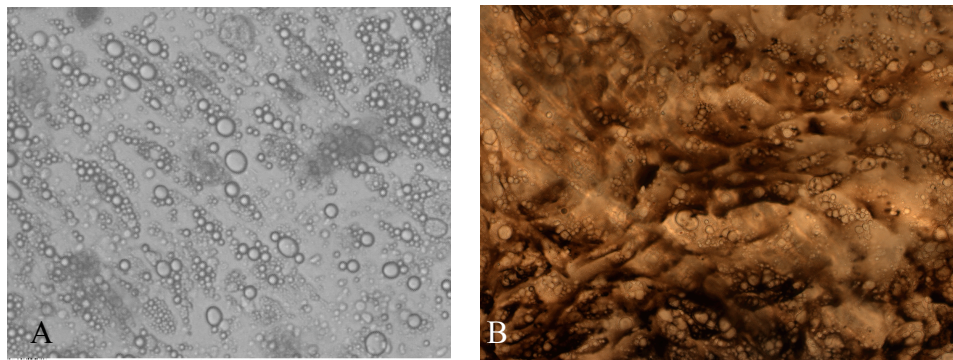
**Figure 3.4. MTP expression in cryosections of the yolk sac of a 15-day-old chicken embryo.**

A and C: Detection of MTP with the  $\alpha$ MTP-B antibody diluted 1:100. Picture C shows an invagination of the yolk sac, which is formed to enlarge its absorptive surface. The reddish-brown staining obtained by the use of biotinylated secondary antibody marks MTP, which is obviously present in the EECs. B and D: Preimmune serum was used instead of the primary antibody as a negative control.

---

#### 3.1.4. Cell culture of and MTP expression in EECs

MTP expression starts at the end of the first trimester of chicken embryonic development and peaks towards the end of the last one. Isolation and culturing of EECs works best around day 9 of embryonic development, due to the yet little amount of lipid droplets present at this stage (Figure 3.5). EECs from older yolk sacs, which already show a higher expression level of MTP, are massively lipid-laden and therefore cannot settle down and adhere onto the culture dish.



**Figure 3.5. Cultured EECs from yolk sacs of 8- to 10-day-old chicken embryos.**

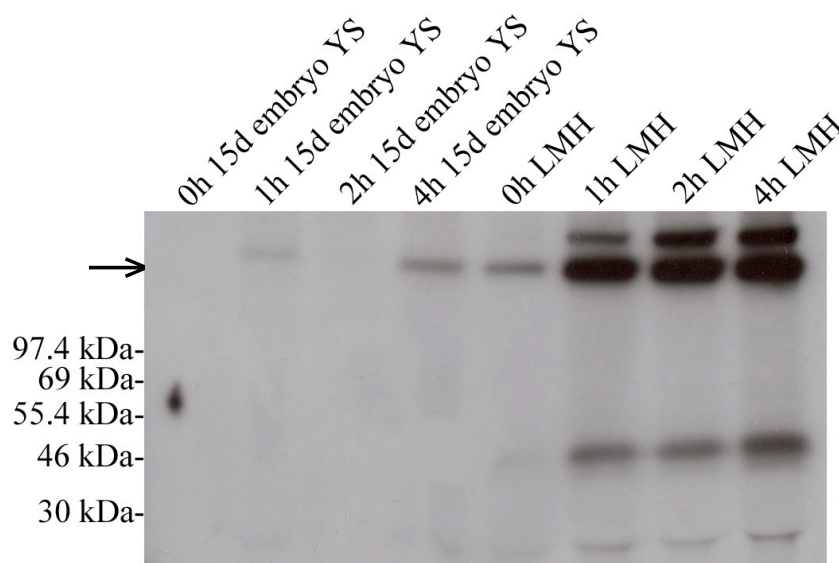
A and B: EECs after 5 days in culture. B: Cysteine lyase activity assay: The presence of cysteine lyase activity and the resulting brown insoluble precipitate prove that the cultured cells actually are EECs.

I improved the method of preparing isolated cells from yolk sac and provided evidence that the cultured cells are in fact EECs, but due to time constraints these cells could not be used for further investigations. Another approach used was organ culture of yolk sacs to show the potential de novo synthesis and secretion of apoB by the yolk sac.

#### 3.1.5. Organ culture and pulse chase analysis

Yolk sac pieces of 0.25 cm<sup>2</sup> size were cultured and used for pulse chase experiments. Newly synthesized apoB100 was immunoprecipitated from the conditioned medium after metabolic labeling and further subjected to SDS-PAGE. Exposition of films to the radioactively labeled proteins led to the findings that apoB100 is indeed synthesized and secreted by cultured yolk sac pieces, although apparently only small amounts (Figure

3.6.). Compared to LMH-cells, a chicken hepatoma cell line that produces apoB-containing lipoproteins (kindly provided by Prof. Dr. N.E. Ivessa), used as a positive control, the quantity of newly synthesized and secreted apoB100 is rather minute, but it cannot be excluded that this might be due to methodic reasons. An optimization of this experiment would be useful to clarify the question of apoB100 de novo synthesis by the yolk sac. Nevertheless, my results indicate that apoB100 is synthesized in vitro by the yolk sac.



**Figure 3.6. Pulse chase analysis with cultured yolk sac pieces.**

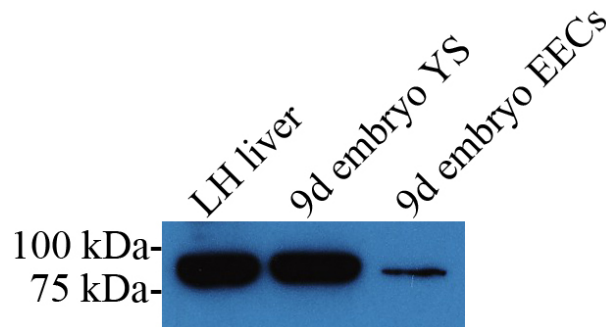
The yolk sac of a 15-day-old embryo was used for organ culture followed by pulse chase analysis and immunoprecipitation of apoB100. Cultured LMH-cells, provided by Prof. Dr. N.E. Ivessa, were used as a positive control. After 1 hour of chase, a weak signal corresponding to apoB is visible, becoming more intensive after 4 hours of chase phase.

### 3.1.6. EEC cell lysate

To demonstrate the expression of MTP in the inner cell layer of the yolk sac, a total cell lysate of isolated EECs of a 9-day-old chicken embryo was prepared, which was further used for western blot analysis. The proteins of the cell lysate from the EECs and membrane protein extracts from the liver of laying hen and the yolk sac of a 9-day-old chicken embryo as controls were separated by SDS-PAGE. According to protein concentrations determined by the Bradford assay, equal amounts of protein of each

---

extract were loaded onto the gel. As the EEC cell lysate is a total protein extract, the amount of MTP in this cell lysate is much lower than in membrane protein extracts of liver and yolk sac.



**Figure 3.7. Western blot analysis of MTP expression in EECs.**

50 µg protein of membrane protein extracts (LH liver, 9d embryo YS) and cell lysate (9d embryo EECs) were loaded onto a 10% gel and separated by SDS-PAGE. After transfer of the proteins to a nitrocellulose membrane, MTP was detected with the  $\alpha$ MTP-B antibody. MTP is expressed in the EECs. The signal obtained in the lane of 9d embryo EECs is much weaker compared to the one of 9d embryo YS. This may be due to the different methods used for preparation of the protein extracts.

By the use of the EECs for making a cell lysate, followed by separation of the proteins, western blot analysis and detection of MTP with a specific antibody, I could verify the presence of MTP in these cells (Figure 3.7). It remains to be excluded, however, that MTP is also expressed in the outer layer of the yolk sac, the mesoderm.

---

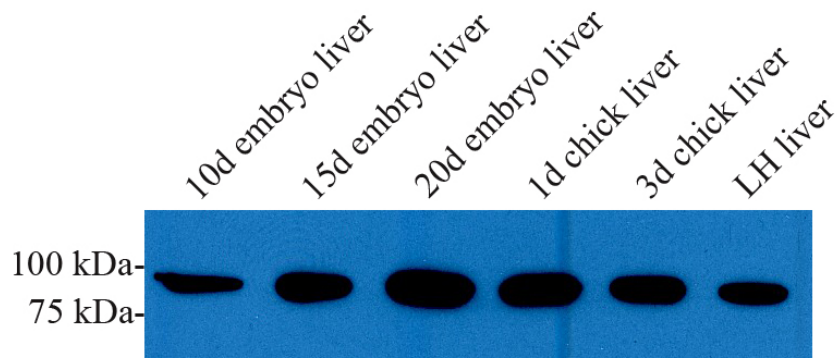
## 3.2. Expression of MTP in chicken embryonic tissues

It is known that MTP is expressed in liver, small intestine, and kidney of the laying hen. Therefore, these tissues of the chicken embryo and hatchling were also targets of my investigations. Due to technical reasons, it is not possible to isolate these organs before day 10 of embryonic development (not yet well-defined and very small). This is the reason, why it was not possible to include liver, small intestine and kidney before day 10 of chicken embryonic development in my experiments.

### 3.2.1. Liver

#### 3.2.1.1. Western blot analysis

Membrane protein extracts of embryonic and adult chicken liver were used to detect MTP therein, using the specific  $\alpha$ MTP-B antibody. As shown in Figure 3.8, MTP is already expressed on day 10 of embryonic development at nearly the same level as in the liver of the laying hen.



**Figure 3.8. Western blot analysis of MTP expression in liver.**

10  $\mu$ g protein of membrane protein extracts of liver of different developmental stages were subjected to a 10% SDS-PAGE, western blot analysis, and detection of MTP using the  $\alpha$ MTP-B antibody. The expression of MTP in liver increases towards the end of embryonic development, followed by a decrease after hatching. Interestingly, the level of MTP expression in liver on day 10 of embryonic development is almost the same as in the liver of laying hen.

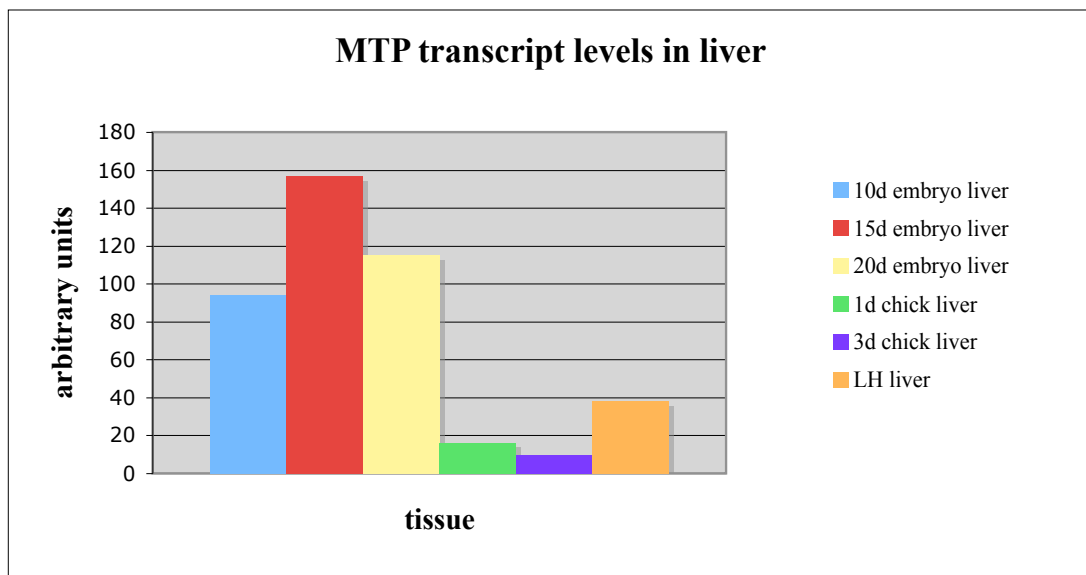


---

Quantification with AlphaEaseFC gave more precise insight into the amounts of MTP expressed at the different developmental stages. Granted that the expression of MTP in the liver of laying hen is 1, its expression level in 10-day-old embryos is 0.95 fold. The level of MTP increases until the end of embryonic development, reaching a 1.46 fold level on day 15 and even a 2.33 fold one on day 20 compared to the amount of MTP in the laying hen's liver. In the 1-day-old hatchling the expression level is 1.83 times higher and in the 3-day-old one 1.49 times, nearly the same as in the 15-day-old embryo.

### 3.2.1.2. qPCR

I also analyzed MTP mRNA levels in liver of different developmental stages. The correlation between protein expression and mRNA level in liver is not that defined as in the yolk sac. The maximum level of mRNA could be detected on day 15 of embryonic development, whereas on day 20 the amount of MTP mRNA is already lower and decreases strongly after hatching, reaching again a higher level in the liver of the full-grown laying hen (Figure 3.9).



**Figure 3.9. Course of MTP transcript levels in the liver of chicken embryos, hatchlings, and laying hen.**

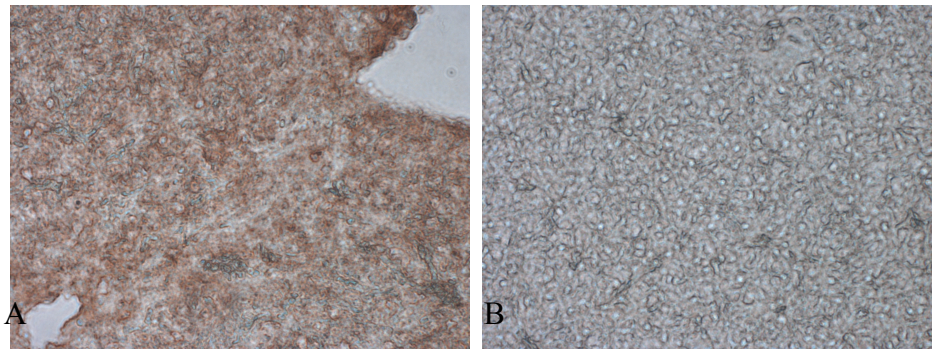
In contrast to the protein level of MTP in liver, which peaks on day 20, the mRNA level reaches its maximum already earlier, i.e. on day 15, suggesting different mechanisms of translational regulation in yolk sac and liver.

---

Interestingly, the MTP transcript level in the liver of 10-day-old chicken embryos is already much higher than in the one of laying hens. This leads to the suggestion that MTP mRNA and protein are already present in liver or primordial hepatocytes before day 10 of embryonic development, possibly from the beginning of the second trimester on (as it is the case in the yolk sac).

#### 3.2.1.3. Immunohistochemistry

Immunohistological analysis should provide further evidence for the presence of MTP in embryonic liver. Paraffin sections were made and the primary  $\alpha$ MTP-B antibody was used for detection of the protein.



**Figure 3.10. MTP expression in paraffin sections of the liver of a 17-day-old chicken embryo.**

A: Detection of MTP with the  $\alpha$ MTP-B antibody diluted 1:100. The reddish-brown staining shows that MTP is expressed in embryonic liver. B. Preimmune serum was used instead of the specific antibody as a negative control.

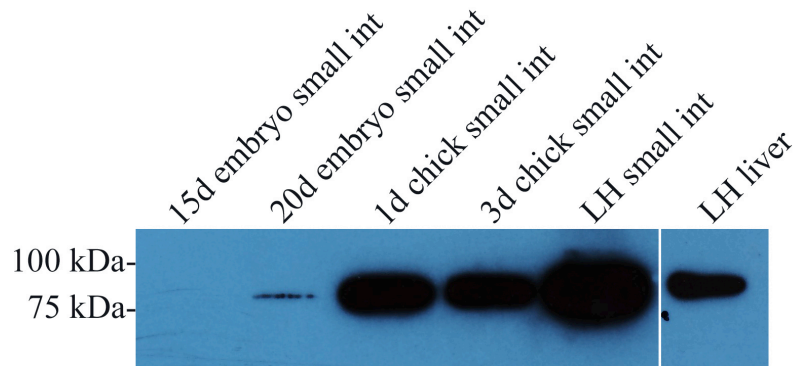
As can be seen in Figure 3.10.A, using the  $\alpha$ MTP-B antibody for the detection, MTP is expressed in embryonic liver. This is in accordance to the results obtained by western blot analysis and quantitative PCR.

---

### 3.2.2. Small intestine

#### 3.2.2.1. Western blot analysis

Membrane protein extracts of the small intestine of chicken embryos and hatchlings were used to investigate the presence of MTP in this tissue. MTP expression in the small intestine seems to start shortly before hatching, and reaches a much higher level in the small intestine of the laying hen than in its liver (Figure 3.11).



**Figure 3.11. Western blot analysis of MTP expression in small intestine.**

10  $\mu$ g of membrane protein extracts of small intestine of different developmental stages and of liver of the laying hen were subjected to a 10% SDS-PAGE and western blot analysis using the  $\alpha$ MTP-B antibody. The expression of MTP obviously starts shortly before hatching, when the chick starts with oral uptake of albumen. In the laying hen, the level of MTP expression in small intestine is significantly higher than in the liver.

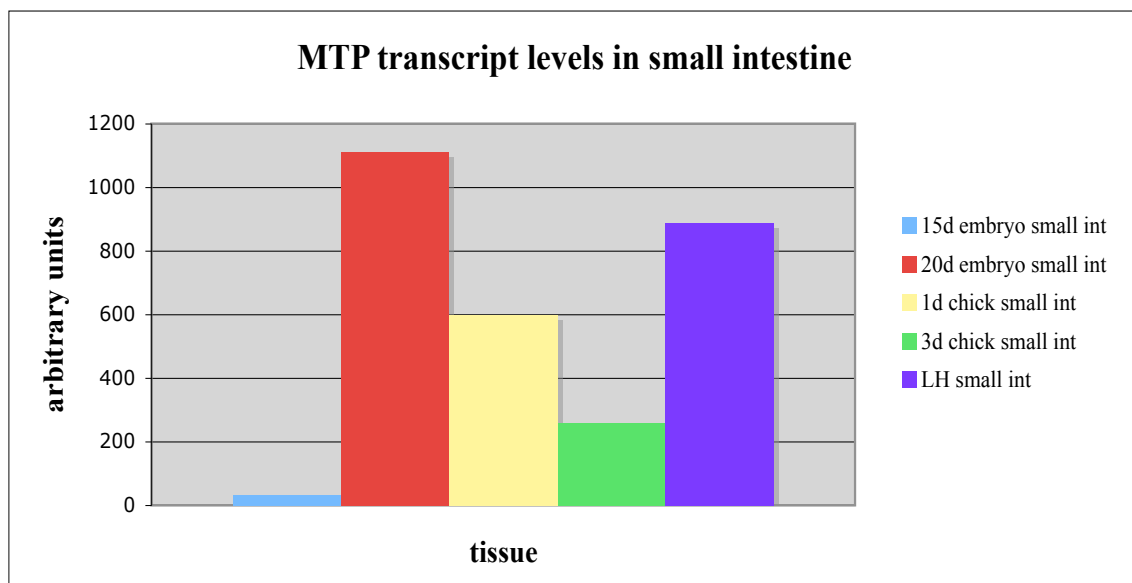
Quantification with AlphaEaseFC showed that the expression of MTP in the small intestine starts in the 20-day-old embryo at a 0.38 fold level compared to the level in the liver of the laying hen. This expression level increases rapidly reaching a around 1.2 fold higher expression in the 1- and 3-day-old chick. In the laying hen, the expression of MTP in small intestine is even 2.4 times higher than in the liver.



---

### 3.2.2.2. qPCR

Analysis of MTP mRNA levels in small intestine of chicken embryos, chicks and laying hen showed that in the 15-day-old embryo, the amount of MTP mRNA in the small intestine is still very low followed by a dramatic increase towards the 20-day-old embryo. Interestingly, the protein level at this time point is still very low, although the mRNA level is higher than in the small intestine of the laying hen. This is another indication for the post-transcriptional regulation of MTP expression or a low translation rate. In the hatchling, the MTP mRNA level decreases and reaches a higher level again in the laying hen (Figure 3.12).



**Figure 3.12. Course of MTP mRNA levels in the small intestine of chicken embryos, hatchlings, and laying hen.**

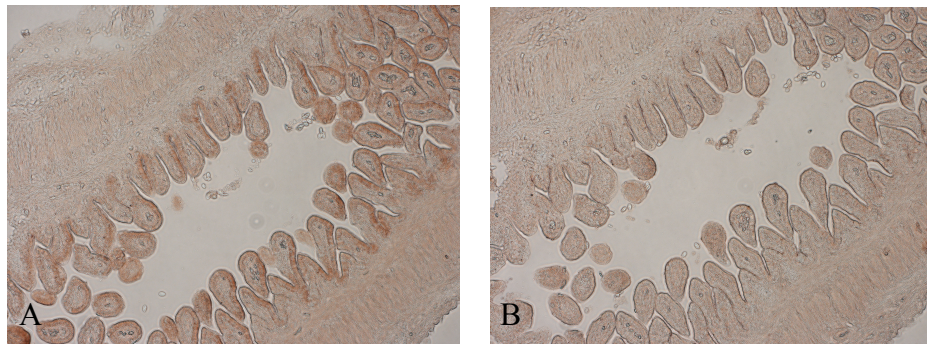
On day 20 of embryonic development, high amounts of MTP mRNA are present in the small intestine. This high level of MTP mRNA is not maintained, it decreases rapidly in the hatchling and returns to a higher level in the laying hen again.

### 3.2.2.3. Immunohistochemistry

Western blot analysis showed that MTP is expressed in the small intestine of chicks from the day 20 of embryonic development on. Using paraffin sections of the small intestine of a 17-day-old chicken embryo, I could show that MTP is already expressed therein at this

---

time point of development. A slight reddish-brown staining is visible in the villi of the small intestine (Figure 3.13).



**Figure 3.13. MTP expression in paraffin sections of the small intestine of a 17-day-old chicken embryo.**

A: MTP was detected using the  $\alpha$ MTP-B antibody diluted 1:100. MTP is localized in the enterocytes lining the villi. B: Preimmune serum was used as a negative control.

Interestingly, MTP could be detected in the small intestine of 17-day-old chicken embryos. Using western blot analysis, there was no MTP expression detected in the small intestine of the 15-day-old embryo. In the small intestine of the 20-day-old chicken embryo, MTP could be detected. Obviously, MTP expression starts at the beginning of the third trimester of embryonic development. During this developmental period, the chicken embryo already orally takes up albumen. Therefore, the intestine already has to be functional, but its full set of skills is not developed until a few days after hatching.

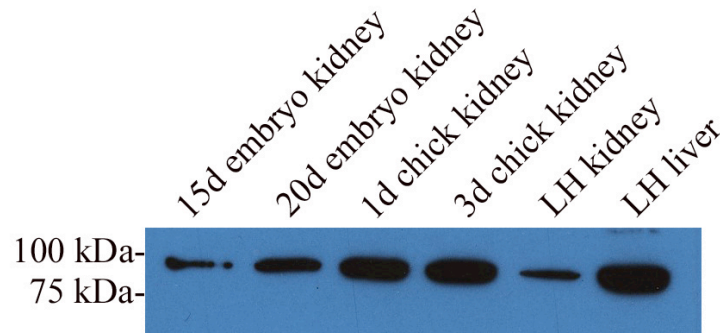
### 3.2.3. Kidney

#### 3.2.3.1. Western blot

Membrane protein extracts from kidneys of chicken embryos, hatchlings, and laying hen were subjected to electrophoretical separation and western blot analysis using the  $\alpha$ MTP-B antibody. MTP is already expressed in kidney at the end of the second trimester of embryonic development, perhaps already earlier. The level of MTP increases continuously from the kidney of the 15-day-old embryo to the 3-day-old chick and

---

probably also thereafter. However, the level of MTP in the kidney of laying hen is much lower than in the hatchlings (Figure 3.14).



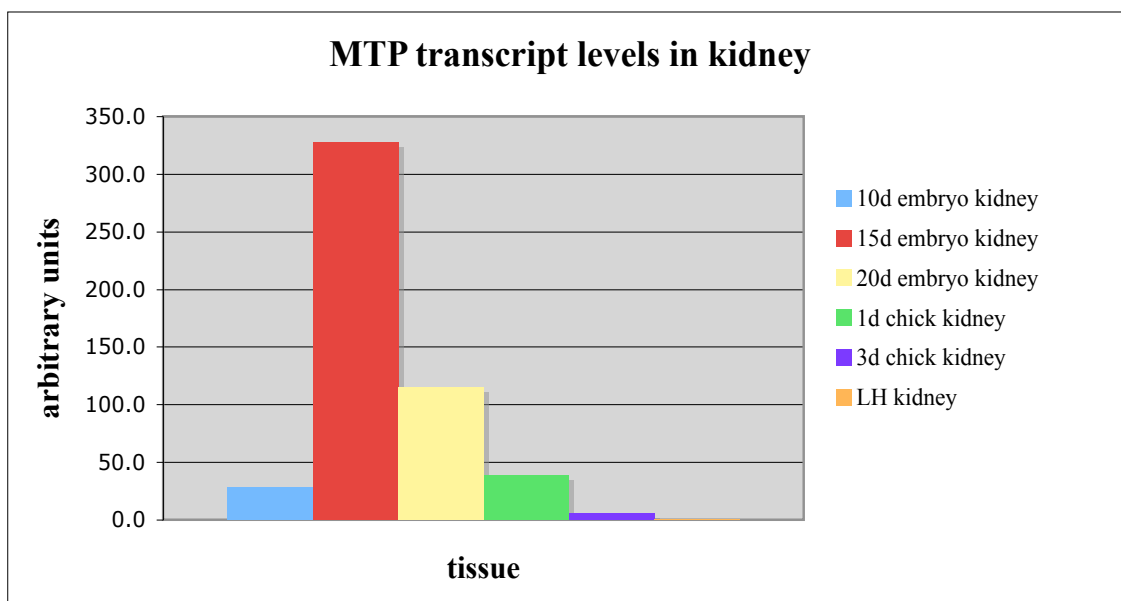
**Figure 3.14. Western blot analysis of MTP expression in kidney.**

25 µg protein of membrane protein extracts of kidney of different developmental stages and of liver of the laying hen were separated using 10% SDS-PAGE and were subjected to western blot analysis using the  $\alpha$ MTP-B antibody. The kidney of 15-day-old embryos already expresses MTP and its expression level increases also after hatching. The level in the kidney of the laying hen is much lower.

This western blot was also analyzed with AlphaEaseFC. The expression level of MTP in the kidney of the 15-day-old embryo is about 0.22 fold of the one in the liver of laying hen. Although the expression level of MTP increases continuously in the developing kidney, at its maximum in the kidney of 3-days-old chicks, it only reaches a 0.88 fold level when compared to the liver of the laying hen. In the kidney of the laying hen, MTP expression level is even only the 0.29 fold of the MTP level in the liver.

#### 3.2.3.2. qPCR

qPCR was also used to analyze MTP transcript levels in the kidney of chicken embryos, hatchlings, and laying hen. Already on day 10 of embryonic development, the level of MTP transcripts in the kidney is already higher than that in the one of the laying hen. On day 15, MTP mRNA level peaks and it continuously decreases thereafter (Figure 3.15). It appears that high levels of MTP expression in kidney are also important before and shortly after hatching, just as in liver and yolk sac.

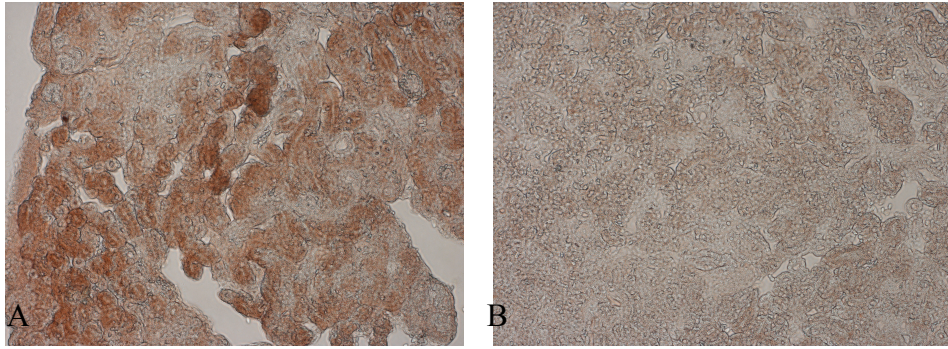


**Figure 3.15. Course of MTP mRNA levels in kidney of chicken embryos, hatchlings, and laying hen.**

MTP mRNA is already present in the kidney of 10-day-old chicken embryos. There is a dramatic increase towards day 15 and a quite rapid decrease thereafter. The MTP transcript level in the kidney of the laying hen is marginal compared to the MTP level therein of other developmental stages.

### 3.2.3.3. Immunohistochemistry

To confirm the presence of MTP in the embryonic kidney, I also did immunohistochemical stainings of kidneys derived from a 17-day-old chicken embryo. The reddish-brown staining can be clearly seen in this tissue (Figure 3.16.A). This is in accordance with the findings obtained by western blotting.



**Figure 3.16. MTP expression in paraffin sections of the kidney of a 17-day-old chicken embryo.**

A:  $\alpha$ MTP-B, diluted 1:100, has been used for the detection of MTP in the kidney. B: Preimmune serum was used as a negative control.

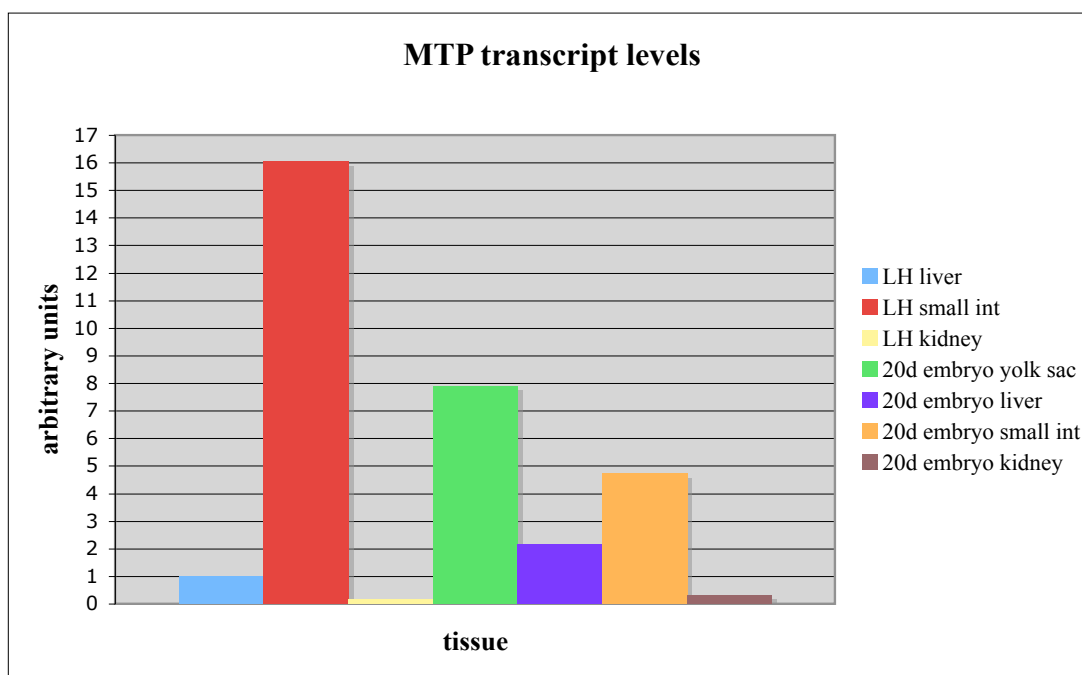
---

### 3.3. MTP mRNA and protein levels in various tissues at different developmental stages of the chicken

Besides the course of MTP transcript and protein levels during embryonic development in yolk sac, liver, small intestine, and kidney, the amounts of MTP mRNA and protein, respectively, in these tissues was also of interest. Transcript levels were investigated using qPCR, protein expression was determined by western blotting and quantifications were performed with AlphaEaseFC.

#### 3.3.1. Transcript levels

qPCR was performed (as described in Materials and Methods 2.6.9) using MTP primers for amplification of MTP transcripts in various tissues at different developmental stages. To enable the comparison of MTP transcript levels in different tissues, the values obtained were normalized to the housekeeping gene ggRS17 (encoding a chicken ribosomal protein). The normalized MTP transcript levels in different tissues are illustrated in Figure 17.



**Figure 3.17. MTP transcript levels in various tissues of different developmental stages.**

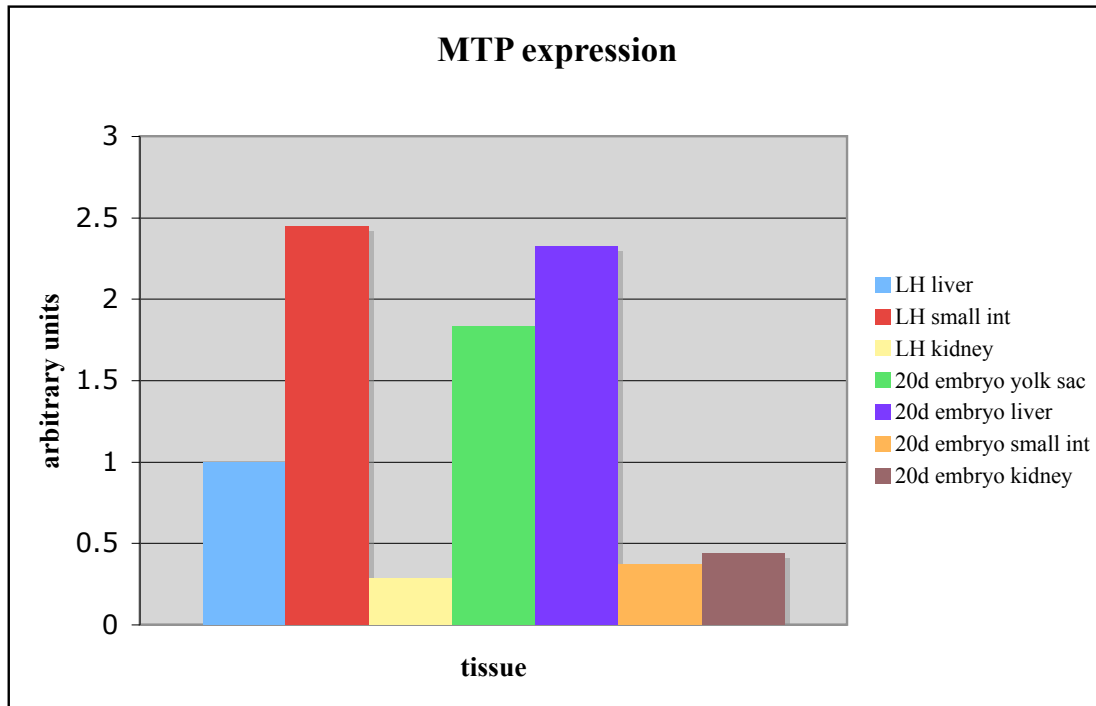
The highest MTP transcript level is observed in the small intestine of the laying hen, followed by the yolk sac and the small intestine of the 20-day-old chicken embryo. The MTP mRNA levels are higher in embryonic liver, than in the adult one. The amounts of MTP transcripts in embryonic and adult kidney are marginal.

As the liver of the laying hen served as an internal standard in every experiment, the normalized MTP transcript level therein is set 1. Compared with laying hen liver, the level in small intestine of the laying hen is 16 times higher and the one of the 20-day-old embryo is 4.7 times higher. In the yolk sac, the MTP mRNA level is also nearly 8 times higher than in the control tissue. Even in the embryonic liver, the MTP mRNA level is twice as high in the liver of the laying hen. In the kidney, the MTP transcript levels reach only a 0.2 fold level in the laying hen and a 0.4 fold in the 20-day-old embryo compared to the liver of the laying hen.

---

### 3.3.2. Protein levels

At the protein level, the amount of MTP in different tissues was determined using AlphaEaseFC, based on western blot analysis. The values obtained were mostly similar to MTP transcript levels, but there were also some remarkable differences in some tissues. For example, in the small intestine of the 20-day-old embryo, the level of MTP mRNA reaches already a very high level, whereas protein expression starts around this time point at a still low level. In liver of the 20-day-old embryo, the amount of MTP mRNA is very low compared to its amount in the liver of the laying hen. At the protein level, the difference between the liver of the embryo and the full-grown animal is not that significant. (Figure 18.)



**Figure 3.18. MTP protein levels in various tissues of different developmental stages.**

At the protein level, the highest MTP expression exists in the small intestine of the laying hen. In contrast to the MTP transcripts, there is a quite high protein level in the liver of the 20-day-old embryo and the level of MTP in the small intestine of the 20-day-old embryo is very low. MTP is expressed at a nearly 2 times higher level in the yolk sac than in the liver of the laying hen. The expression of MTP in kidney is only half as high in the 20-day-old embryo and even lower in the laying hen.



---

## 4. Discussion

MTP plays a major role in the formation of apoB-containing lipoproteins and therefore it is an indispensable protein in lipid metabolism. In humans, MTP is expressed mainly in the liver and small intestine, but also in kidney, ovary, and testis (Shoulders et al. 1993). Later studies showed that MTP is also expressed in human yolk sac and placenta (Madsen et al. 2004). In mouse, MTP is also expressed in liver and small intestine of both embryos and adult animals, and in yolk sac (Nakamuta et al. 1996; Shelton et al. 2000). In the laying hen, MTP is present in liver, small intestine, and kidney, an organ, which plays a major role in chicken lipoprotein metabolism (Ivessa et al. in revision; Walzem et al. 1999).

The presence of MTP in organs involved in lipid metabolism in the chicken and in the yolk sac of rodents and humans lead to the assumption that MTP might also be present in the yolk sac of chicken embryos, as well as in other tissues. The chicken embryo depends on the yolk sac because it nourishes on the nutrients and lipids present therein (Noble and Cocchi. 1990). Lipids have to be transported from the yolk to the growing embryo. This process requires proteins involved in lipid metabolism and transport (Speake et al. 1998). MTP probably forms part of the complex interplay of a multitude of proteins participating in the uptake, remodeling, and secretion of lipoproteins on their way from

---

the yolk to the embryonic circulation (Hermann et al. 2000). It is also supposed to play a role in chicken embryonic lipid metabolism in various organs. Therefore, my studies were focused on the role of MTP during chicken embryonic development.

The chicken yolk sac was the tissue of major interest, as it is responsible for providing the growing embryo with nutrients that are required for development and growth. I could show that MTP is expressed in the chicken yolk sac from the end of the first trimester of embryonic development on (Figure 3.1). This result is similar to the findings of MTP mRNA expression in the mouse yolk sac. At day 9.5 of mouse embryonic development, which was the earliest time point examined, the yolk sac already showed stable MTP expression (Shelton et al. 2000).

As can be seen in Figure 3.1 and 3.2, the amount of MTP in the chicken yolk sac increases continuously until the end of embryonic development and rapidly decreases after hatching. Due to the presence of apoB-fragments in the yolk and apoB100 in the embryonic blood system, it has been suggested that lipoproteins are re-synthesized in the yolk sac (Hermann et al. 2000) and the presence of MTP therein gives support to this hypothesis. The huge amount of MTP in the yolk sac during the third trimester of embryonic development probably is a consequence of the massive uptake of lipids and nutrients from the yolk sac during this time period (Speak et al. 1998).

Similarly, MTP mRNA is present in the yolk sac and its amounts increase and decrease simultaneously with the protein levels, as shown in Figure 3.3. It appears that the MTP mRNA is translated rapidly without complex regulatory mechanisms that inhibit or delay the expression of functional MTP.

Localization of MTP in the yolk sac was also of interest during my diploma thesis. It has been supposed that MTP is present and functional in the EECs (Hermann et al. 2000). These cells form the inner cell layer of the yolk sac, which is in direct contact with the yolk. Therefore, I wanted to verify the presence of MTP in this cell layer. This was done by cryosections (Figure 3.4) and also by western blot analysis of a cell lysate of isolated EECs (Figure 3.7). These two methods showed that MTP is present in the EECs. To investigate, whether MTP is involved in apoB-containing lipoprotein synthesis in this cell

---

layer of the yolk sac, primary cell culture of EECs was established. This is a very time-consuming process, and therefore it could not be pursued extensively. There could, however, evidence be provided that the cultured cells are in fact EECs (Figure 3.5). To demonstrate, that the yolk sac also secretes apoB-containing lipoproteins, organ culture of yolk sac pieces was performed. Using pulse chase analysis, it could be show, that apoB100 is synthesized and secreted by the yolk sac, a process that requires MTP (Figure 3.6).

In the laying hen, MTP is expressed in liver, small intestine, and kidney (Ivessa et al. in revision). Therefore, embryonic liver, small intestine, and kidney were also tissues of interest for this study. Western blot analysis, quantitative PCR, and paraffin sections were used to analyze the expression of MTP in these tissues during chicken embryonic development.

As shown in Figure 3.8, in the embryonic liver, MTP expression starts most likely around day 10 of chicken embryonic development. It cannot be determined definitely, at which time MTP expression starts, because it is not possible to isolate liver nor other organs of chicken embryos before day 10. As the expression level is already quite high in the liver of the 10-day-old chicken embryo (0.95 fold of the level observed in the liver of the laying hen), it is suggested that MTP expression starts already earlier, perhaps at the end of the first trimester of embryonic development, as it is the case in yolk sac. The level of MTP in the embryonic liver increases continuously until the end of embryonic development and decreases again after hatching. This is a very similar course of MTP expression compared to the one in the yolk sac.

The liver is the major organ of lipid metabolism in the chicken. Lipoproteins, secreted from the yolk sac into the embryonic circulation, enter the embryo via the portal vein, first pass the liver, and then supply the other organs. Lipids not needed by the other organs, return to the liver and accumulate therein. As most lipoproteins and lipids enter the embryonic circulation during the third trimester of embryonic development, there is also the highest rate of their accumulation in the embryonic liver during this time period. The liver's attempt to get rid of excess lipids may lead to a higher expression of MTP,

---

trying to increase the formation of apoB-containing lipoproteins, and the subsequent higher secretion of lipids. (Noble and Cocchi. 1990)

At the mRNA level, MTP is transcribed at a quite high level already in the liver of the 10-day-old embryo (Figure 3.9). This level further increases until day 15 of embryonic development and decreases thereafter. Shortly after hatching, it even reaches a lower level than in the liver of the laying hen. The mRNA level of MTP in the liver peaks earlier in embryonic development than the protein level and it also decreases more drastically than the amount of protein. This could indicate that the MTP mRNA is somehow posttranscriptionally regulated in the embryonic liver.

In the mouse, MTP mRNA is also expressed already at day 9.5 of embryonic development in the hepatic primordium. MTP expression also increases from thereon. (Shelton et al. 2000)

Immunohistochemical analysis of MTP expression in chicken embryonic liver confirmed the presence of MTP in this tissue (Figure 3.10).

Western blot analysis of protein extracts from chicken small intestine (Figure 3.11) showed that MTP is expressed therein from around day 20 of embryonic development on. Apparently, MTP expression in small intestine starts in the third trimester of embryonic development, when the chick starts with the oral uptake of albumen food. Interestingly, the expression level of MTP in the small intestine is already very high one day after hatching compared to the laying hen and in the laying hen, the level of MTP in the small intestine is a multiple of the one in the liver.

In the small intestine of humans, MTP is involved in the synthesis of apoB48-containing lipoproteins (chylomicrons), but chicken lack this type of lipoprotein (Black 2007; Tarugi et al. 1990). It is, however, possible that MTP in small intestine is required for the assembly of so-called portomicrons. These are intestinally derived lipoproteins, which carry apoB100 (Steinmetz et al. 1998).

At the mRNA level, it was determined that MTP is transcribed already before hatching in the small intestine. Little amounts of MTP transcripts can already be detected in the small intestine of the 10-day-old chicken embryo. At day 15 of embryonic development, the

---

MTP mRNA level peaks and decreases thereafter, reaching again a somewhat higher level in the laying hen. (Figure 3.12) As in the liver, it could be that MTP is regulated post-transcriptionally.

In mouse, MTP mRNA has been detected in the small intestine of embryos at developmental stage 12.5. At developmental stage 16.5, MTP expression is strongly upregulated in the small intestine and maintains a high level also post-natal and during adulthood. (Shelton et al. 2000)

Using immunohistochemistry, it could be shown, that MTP is present in small amounts in the villi of the small intestine of 17-day-old chicken embryos (Figure 3.13).

The avian kidney is also involved in lipid metabolism (Walzem et al. 1999). As MTP is expressed in the kidney of the laying hen, the embryonic kidney was also of interest for my study. I could show that MTP is expressed at day 15 and later on in the embryonic kidney, perhaps already earlier. It is not possible to determine the exact time point when MTP expression starts, due to the small size of embryonic kidney. Interestingly, the course of MTP expression in the kidney is quite different to the ones in yolk sac and liver. The MTP level in the kidney continuously increases from day 15 of embryonic development to the 3-day-old chick. In the kidney of the laying hen, MTP is expressed at a lower level again. (Figure 3.14)

At the mRNA level, the highest amount of MTP transcript in the kidney was observed at day 15 of chicken embryonic development, and it decreases dramatically thereafter, as can be seen in Figure 3.15. This is quite in contrast to the course of MTP expression in the kidney, but as the level of MTP in the kidney is very low compared to the other tissues where this protein is expressed, it might be regulated differently in the kidney leading to a large time interval between transcription and translation.

In mouse embryos, neither in early developmental stages nor in later ones MTP mRNA could be detected (Shelton et al. 2000).

Immunohistochemical analysis of chicken embryonic kidney emphasized the presence of MTP therein and also supports the notion that MTP plays a role in this organ (Figure 3.16).

---

To compare the amounts of MTP protein and mRNA in the different tissues to each other, western blot analysis and quantitative PCR experiments were performed. At the mRNA level, the highest level of MTP transcripts is observed in the small intestine of the laying hen. Compared to this transcript level, the amount of MTP mRNA in the liver and the kidney of the laying hen are marginal. (Figure 3.17)

Similar results were obtained in the mouse. Around embryonic developmental stage 16.5, the MTP mRNA level in the liver surmounts the level in the small intestine. From there on, the higher MTP level in the small intestine is maintained until post-natal day 1.5 and also in adulthood. (Shelton et al. 2000)

In the 20-day-old chicken embryo, the highest level of MTP transcript is present in the yolk sac, followed by the small intestine, liver, and finally kidney. The difference between the levels in the different tissues is not that high in the embryo when compared to the same tissues in the laying hen. (Figure 3.17)

At the protein level, a similar distribution of MTP is observed in the different organs of the laying hen compared to the mRNA levels in the respective tissues. The highest level of MTP protein in the laying hen is detected in the small intestine, followed by the liver and the kidney. In the 20-day-old embryo, the situation is somewhat different at the protein level compared to the MTP mRNA level. The highest level of MTP is found in the liver, followed by the yolk sac. The smallest amounts of MTP are present in the kidney and the small intestine of the 20-day-old chicken embryo. (Figure 3.18)

The results of this study clearly showed that MTP is expressed during chicken embryonic development in liver, kidney, small intestine, and yolk sac. Expression of MTP starts in the yolk sac presumably at the end of the first trimester of embryonic development. It could be shown that MTP is expressed in liver and in kidney shortly later. It cannot be determined, whether MTP is expressed in these organs already at an earlier developmental stage, because isolation of organs earlier in embryonic development is not possible. MTP expression in the small intestine starts around day 17 of embryonic development. Studies with mice showed a similar expression profile of MTP in yolk sac, liver, and small intestine. Initially, MTP expression starts in the mouse yolk sac, followed

---

by the primordial liver and subsequently in the developing small intestine (Shelton et al. 2000). MTP in the yolk sac is supposed to be involved in apoB re-synthesis (Hermann et al. 2000), and the findings of this study also give support to this suggestion. Liver, small intestine, and kidney are organs involved in lipid-metabolism, and therefore also require functional MTP, especially during embryonic development.

Future plans to extend this study include cultivation of primary EECs. It should then be possible to determine if these particular cells re-synthesize and secrete apoB100-containing lipoproteins. These cells could also be used for immunofluorescent staining of MTP and other proteins involved in uptake, degradation, and re-synthesis of lipoproteins to localize this process. Cryosections of the yolk sac stained with a fluorescently labeled secondary antibody could give information about the exact localization of MTP in polarized EECs. As MTP is involved in apoB biogenesis, it could be that it is localized in the ER close to the basal side of the EECs, where apoB is secreted to the embryonic blood system, rather than near the apical cellular membrane.

To investigate whether MTP in liver and kidney is expressed already before day 10 of embryonic development, cryosections of chicken embryos could be used to detect MTP in these organs.

As the protein level of MTP in the 3-day-old chick in liver, small intestine, and kidney is not the same as in the laying hen, it would also be quite interesting, at which time point of development, the expression level of MTP adjusts to its final level. This might occur shortly after hatching, or later on during sexual maturation, as estrogen and testosterone influence lipid metabolism. On the other hand, there has no effect of estrogen been found on the level and activity of MTP in mature animals and in a cell culture system using LMH-2A cells (Ivessa et al. in revision). Further, it would be interesting to study the influence of sex-specific hormones on MTP expression, especially during the laying period of laying hens compared to roosters.

---

## Bibliography

- Black, D.D. 2007. Development and physiological regulation of intestinal lipid absorption. I. Development of intestinal absorption: cellular events in chylomicron assembly and secretion. *Am. J. Physiol. Gastrointest. Liver. Physiol.* 293: G519-G524
- Dixon, J.L., S. Furukawa, and H.N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* 266: 5080-5086
- Gordon, D.A. and H. Jamil. 2000. Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. *Biochim. Biophys. Acta* 1486: 72-83
- Hagan, L.H., B. Kienzle, H. Jamil, and N. Hariharan. 1994. Transcriptional regulation of human and hamster microsomal triglyceride transfer protein genes. *J. Biol. Chem.* 269: 28737-28744
- Hermann, M., M.G. Mahon, K.A. Lindstedt, J. Nimpf, and W.J. Schneider. 2000. Lipoprotein receptors in extraembryonic tissues of the chicken, *J. Biol. Chem.* 275: 16837-16844



- 
- Herz, J. and R.V. Farese, Jr. 1999. The LDL receptor gene family, apolipoprotein B and cholesterol in embryonic development. *J. Nutr.* 129: 473S-475S
- Hussain, M.M. and A. Bakillah. 2008. New approaches to target microsomal triglyceride transfer protein. *Curr. Opin. Lipid.* 19:572-578
- Hussain, M.M., J. Iqbal, K. Anwar, P. Rava, and K. Dai. 2003. Microsomal triglyceride transfer protein: a multifunctional protein. *Front. Biosci.* 8: 500-506
- Hussain, M.M., J. Shi, P. Dreizen. 2003. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* 44:22-32
- Hussain, M.M., P. Rava, X. Pan, K. Dai, S.K. Dougan, J. Iqbal, F. Lazare, and I. Khatun. 2008. Microsomal triglyceride transfer protein in plasma and cellular lipid metabolism. *Curr. Opin. Lipid.* 19: 277-284
- Ivessa, N.E., E. Rehberg, B. Kienzle, F. Seif, R. Hermann, M. Hermann, W.J. Schneider, and D.A. Gordon. 2009. Molecular cloning, expression and hormonal regulation of the chicken microsomal triglyceride transfer protein, in revision
- Lin, M.C.M., C. Arbeeny, K. Bergquist, B. Kienzle, D.A. Gordon, and J.R. Wetterau. 1994. Cloning and regulation of hamster microsomal triglyceride transfer protein. *J. Biol. Chem.* 269: 29138-29145
- Madsen, E.M., M.L.S. Lindegaard, C.B. Andersen, P. Damm, and L.B. Nielsen. 2004. Human placenta secretes apolipoprotein B-100-containing lipoproteins. *J. Biol. Chem.* 279: 55271-55276
- Moestrup, S.K. and L.B. Nielsen. 20005. The role of the kidney in lipid metabolism. *Curr. Opin. Lipidol.* 16: 301.306
- Moran, E. T. Jr. 2007. Nutrition of the developing embryo and hatchling. *Poult. Sci.* 86: 1043-1049
- Noble, R.C. and M. Cocchi. 1990. Lipid metabolism and the neonatal chicken, *Prog. Lipid Res.* 29: 107-140

- 
- Nakamuta, M., B.H. Chang, R. Hoogeveen, W.H. Li, L. Chan. 1996. Mouse microsomal triglyceride transfer protein large subunit: cDNA cloning, tissue-specific expression and chromosomal localization. *Genomics* 33: 313-316
- Pan, M., J. Liang, E.A. Fisher, and H.N. Ginberg. 2002. The late addition of core lipids to nascent apolipoprotein B100, resulting in the assembly and secretion of triglyceride-rich lipoproteins, is independent of both microsomal triglyceride transfer protein activity and new triglyceride synthesis. *J. Biol. Chem.* 277: 4413-4421
- Raabe, M., L.M. Flynn, C.H. Zlot, J.S. Wong, M.M. Véniant, R.L. Hamilton, and S.G. Young. 1998. Knockout of the abetalipoproteinemia gene in mice: Reduced lipoprotein secretion in heterozygous and embryonic lethality in homozygous. *Proc. Natl. Acad. Sci.* 95: 8686-8691
- Shelness, G.S. and A.S. Ledford. 2005. Evolution and mechanism of apolipoprotein B-containing lipoprotein assembly. *Curr. Opin. Lipid.* 16: 325-332
- Shelton, J.M., M. Lee, J.A. Richardson, and S.B. Patel. 2000. Microsomal triglyceride transfer protein expression during mouse development. *J. Lipid Res.* 41: 532-537
- Shoulders, C.C., D.J. Brett, J.D. Bayliss, T.M. Narcisi, A. Jarmuz, T.T. Grantham, P.R. Leoni, S. Bhattacharya, R.J. Pease, P.M. Cullen. 1993. Abetalipoproteinemia is caused by defects of the gene encoding the 97 kDa subunit of a microsomal triglyceride transfer protein. *Hum. Mol. Genet.* 2: 2109-2116
- Smolenaars, M.M.W., O. Madsen, K.W. Rodenburg, and D.J. van de Horst. 2007. Molecular diversity and evolution of the large lipid transfer protein superfamily. *J. Lipid Res.* 48: 489-502
- Speak, B. K., A.M.B. Murray, and R.C. Noble. 1998. Transport and transformations of yolk lipids during development of the avian embryo. *Prog. Lipid Res.* 37: 1-32
- Steinmetz, A., M. Hermann, J. Nimpf, R. Aebersold, A. Ducret, R.B. Weinberg, and W.J. Schneider. 1998. Expression and conservation of apolipoprotein AIV in an avian species. *J. Biol. Chem.* 273: 10543-10549
- Tarugi, P., L. Albertazzi, S. Nicolini, and S. Calandra. 1990. Absence of apolipoprotein B-48 in the chick, *Gallus domesticus*. *J. Lipid Res.* 31: 417-427
-

- 
- Tarugi, P., S. Nicolini, L. Marchi, G. Ballarini, and S. Calandra. 1994. Apolipoprotein B-100 production and cholesteryl ester content in the liver of developing chick. *J. Lipid Res.* 35: 2019-2031
- Tarugi, P., M. Aversa, E. di Leo, A.B. Cefalù, D. Noto, L. Magnolo, L. Cattin, S. Bertolini, S. Calandra. 2007. Molecular diagnosis of hypobetalipoproteinemia: An ENID review. *Atherosclerosis* 195: e19-e27
- Terasawa, Y., S.J. Cases, J.S. Wong, H. Jamil, S. Jothi, M.G. Traber, L. Packer, D.A. Gordon, R.L. Hamilton, and R.V. Farese, Jr. 1999. Apolipoprotein B-related gene expression and ultrastructural characteristics of lipoprotein secretion in mouse yolk sac during embryonic development. *J. Lipid Res.* 40: 1967-1977
- Walzem, R.L., R.J. Hansen, D.L. Williams, and R.L. Hamilton. 1999. Estrogen induction of VLDL assembly in egg-laying hens. *J. Nutr.* 129: 467S-472S

---

## List of figures

Figure 1.2. Structural and functional domains of the M-subunit of MTP. ....	11
Figure 1.3. Lipid transfer activity. ....	12
Figure 1.4. ApoB binding activity. ....	13
Figure 1.5. Membrane binding activity.....	13
Figure 1.6. Ovary and oviduct of the laying hen .....	21
Figure 1.7. 10-day-old chicken embryo.....	22
Figure 2.1. Genetic map of the pCR 2.1-TOPO cloning vector .....	28
Figure 3.1. Western blot analysis of MTP expression in the chicken yolk sac. ....	53
Figure 3.2. Quantification of MTP expression in the yolk sac with AlphaEaseFC.....	54
Figure 3.3. Course of MTP transcript levels in the yolk sac of chicken embryos and hatchlings. ....	55
Figure 3.4. MTP expression in cryosections of the yolk sac of a 15-day-old chicken embryo. ....	56
Figure 3.5. Cultured EECs from yolk sacs of 8- to 10-day-old chicken embryos.....	57
Figure 3.6. Pulse chase analysis with cultured yolk sac pieces. ....	58
Figure 3.7. Western blot analysis of MTP expression in EECs.....	59
Figure 3.8. Western blot analysis of MTP expression in liver.....	60

---

Figure 3.9. Course of MTP transcript levels in the liver of chicken embryos, hatchlings, and laying hen. ....	61
Figure 3.10. MTP expression in paraffin sections of the liver of a 17-day-old chicken embryo. ....	62
Figure 3.11. Western blot analysis of MTP expression in small intestine.....	63
Figure 3.12. Course of MTP mRNA levels in the small intestine of chicken embryos, hatchlings, and laying hen.....	64
Figure 3.13. MTP expression in paraffin sections of the small intestine of a 17-day-old chicken embryo. ....	65
Figure 3.14. Western blot analysis of MTP expression in kidney. ....	66
Figure 3.15. Course of MTP mRNA levels in kidney of chicken embryos, hatchlings, and laying hen.....	67
Figure 3.16. MTP expression in paraffin sections of the kidney of a 17-day-old chicken embryo. ....	68
Figure 3.17. MTP transcript levels in various tissues of different developmental stages.	70
Figure 3.18. MTP protein levels in various tissues of different developmental stages. ....	71

---

## List of tables

Table 2.1. Properties of chemically competent <i>E.coli</i> TOP10 bacteria .....	27
Table 2.2. Properties of T/A cloning vector .....	28
Table 2.3. List of oligonucleotide primers used for PCR .....	29
Table 2.4. List of antibodies .....	30
Table 2.5. Standard mixture of chemicals and enzymes used for PCR .....	31
Table 2.6. Standard conditions used for PCR .....	31
Table 2.7. Standard mixture of chemicals and enzymes for qPCR .....	35
Table 2.8. Standard program used for qPCR with chicken MTP primers. ....	36
Table 2.9. Composition of SDS-gels .....	40

---

## Abbreviations

µg	.....	microgram
µl	.....	microliter
A	.....	adenine
aa	.....	amino acid
AEC	.....	3-amino-9-ethylcarbazole
Amp	.....	ampicillin
apo	.....	apolipoprotein
APS	.....	ammoniumpersulfate
AP-1	.....	activator protein 1
bp	.....	base pair
BSA	.....	bovine serum albumin
C	.....	cytosine, Celsius
C-terminal	....	carboxy-termial
cDNA	.....	complementary DNA
CE	.....	cholesterol ester
CD1d	.....	cluster of differentiation 1d
d	.....	day
DMEM	.....	Dulbecco's Modified Eagle Medium

---

DMSO .....	dimethylsulfoxide
DNA .....	desoxyribonucleic acid
DTT .....	dithiothreitol
dNTP .....	deoxyribonucleotide triphosphate
EEC .....	endodermal epithelial cell
ECL .....	enhanced chemiluminescence
EDTA .....	ethylenediaminetetraacetic acid
ER .....	endoplasmatic reticulum
FCS .....	fetal calf serum
fw .....	forward
g .....	gram
G .....	guanine
gg .....	Gallus gallus
gge .....	gauge
h .....	hour
HBSS .....	Hanks' balanced salt solution
HDL .....	high density lipoprotein
HMG .....	3-hydroxy-3-methyl-glutaryl
HNF .....	hepatocyte-specific trans-factor
HRP .....	horse radish peroxidase
int .....	intestine
Kan .....	kanamycin
kb .....	kilobase
kDa .....	kilodalton
l .....	liter
LB .....	Luria Bertani
LDL .....	low density lipoprotein
LH .....	laying hen
LLT .....	large lipid transfer
LLTP .....	large lipid transfer protein
LMH .....	leghorn male hepatoma



---

LPL .....	lipoprotein lipase
LXR .....	liver X receptor
M .....	molar
mA .....	milliampere
mg .....	milligram
Mg .....	magnesium
min .....	minute
ml .....	milliliter
mM .....	millimolar
mRNA .....	messenger RNA
MTP .....	microsomal triglyceride transfer protein
M-subunit ....	microsomal triglyceride transfer protein-subunit
N-linked .....	asparagine-linked
nm .....	nanometer
NK .....	natural killer
N-terminal ...	amino terminal
N terminus ...	amino terminus
OD .....	optical density
PAA .....	polyacrylamide
PAGE .....	polyacrylamide gelelectrophoresis
PBS .....	phosphate buffered saline
PCR .....	polymerase chain reaction
PDI .....	protein disulfide isomerase
PFA .....	paraformaldehyde
pmol .....	picomolar
P-subunit .....	protein disulfide isomerase-subunit
qPCR .....	quantitative PCR
rev .....	reverse
RNA .....	ribonucleic acid
RNase .....	ribonuclease
rpm .....	rotations per minute

---

RPMI ..... Roswell Park Memorial Institute  
sec ..... second  
SDS ..... sodium dodecylsulfate  
SR-BI ..... scavenger receptor class B type I  
SRE ..... sterol responsive element  
T ..... thymine, tween  
TBS ..... tris bufferd saline  
TEMED ..... N,N,N,'N' tetramethylethylendiamine  
Tris ..... trishydroxymethylaminomethan  
U ..... unit  
UV ..... ultraviolet  
V ..... volt  
VLDL ..... very low density lipoprotein  
Vtg ..... vitellogenin  
X-Gal ..... 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside  
YS ..... yolk sac

---

## Curriculum vitae

Name: Christine Eresheim  
Family status: unmarried, no children  
Date of birth: July 19, 1985, Oberpullendorf, Austria  
Nationality: Austria  
Current address: Gumpendorferstraße 83-85/1/51, A-1060 Vienna  
Mobile: +43 664 5066552  
E-mail: christine.eresheim@gmx.at

### Education

09. 2008 – 06. 2009 Master thesis at the Medical University of Vienna, Department of Medical Biochemistry, Dr. Bohrgasse 9, A-1030 Vienna. Subject: “Characterization of MTP during chicken embryonic development”
10. 2003 – 11. 2008 Undergraduate Studies in Molecular Biology, Faculty of Life Sciences, University of Vienna, Austria

---

## **Primary education**

06. 2003                      School leaving examination “summa cum laude”
09. 1995 – 06.2003      BG, BRG, BORG Oberpullendorf, Austria, with main focus on foreign languages
09. 1991 – 06. 1995      Elementary school in Piringsdorf, Austria

## **Employment**

07. 2004 – 08. 2007      Summer jobs at Bogner Veranstaltungen GmbH, Wiesen, Austria

## **Language skills**

German native language  
English fluent oral and written  
Spanish oral and written  
French good knowledge

## **Computer skills**

MS-Office  
Adobe Photoshop  
Online databases  
Experienced with the user interfaces of Mac OS X and Windows

---

**Scientific skills**

Detailed knowledge of microbial test methods

Detailed knowledge of RNA, DNA, and protein isolation and analysis

Skilled in the use of laboratory instrumentation related to microbiology, cellular, and molecular genetics

Skilled in performing scientific experiments and in interpreting the results